ANAEROBIC BIODEGRADATION OF A NAPHTHENIC ACID UNDER DENITRIFYING CONDITIONS

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ABSTRACT

Oil sand deposits in the Athabasca Basin in Alberta represent one of the largest global oil reserves. The bitumen contents of oil sand shallow deposits are recovered by surface mining using modified version of the Clark hot water process. Extraction of bitumen results in extremely large volumes of process water, which are contaminated with naphthenic acids. Various ex-situ treatment techniques including ozonation, advanced oxidation, adsorption, and bioremediation have been evaluated for the treatment of these waters. Previous studies conducted by Paslawski *et al.* (2009) investigated aerobic biodegradation of naphthenic acids in properly designed and carefully operated bioreactors. In the current work, anaerobic biodegradation of naphthenic acids under denitrifying condition was examined as a potential approach to eliminate the aeration cost in ex-situ treatment and as an alternative for application of in-situ treatment of oil sand process water in stabilization ponds was examined. Using trans-4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA), a microbial mixed culture developed in earlier works (Paslawski et al., 2009), and nitrate as an electron acceptor, anaerobic biodegradation of trans-4MCHCA were studied in batch and continuous bioreactors: continuous stirred tank reactor (CSTR) and biofilm system. Effects of naphthenic acid concentration, temperature, and loading rate on biodegradation process were investigated.

The batch studies showed that initial concentration of trans-4MCHCA influenced the biodegradation rate where the increase in initial concentration of trans-4MCHCA from 100 to 250 mg L^{-1} led to a higher rate but further increase in concentration did not have a marked effect. Moreover, batch experiments at temperatures ranging from 10° to 35°C demonstrated that the

optimum temperature was in the range of 20 - 24°C. Continuous anaerobic biodegradation in the CSTR showed that increase in loading rate of trans-4MCHCA caused an increase in removal rate of both trans-4MCHCA and nitrate. Rates were decreased as the system approached the cell washout. The maximum biodegradation rate and nitrate removal rate, achieved at trans-4MCHCA loading rate of 157.8 mg L^{-1} h^{-1}, were 105.4 mg L^{-1} h^{-1} and 144.5 mg L^{-1} h^{-1}, respectively. A similar dependency between the loading and removal rates was also observed in the biofilm reactor. The maximum removal rate of trans-4MCHCA and nitrate in the biofilm reactor, operated at room temperature (24 ± 2ºC) were 2,028.1 mg L^{-1} h^{-1} and 3,164.7 mg L^{-1} h^{-1}, respectively and obtained at trans-4MCHCA loading rate of 2,607.9 mg L^{-1} h^{-1}.

Comparison of the results from aerobic batch systems obtained by Paslawski *et al.* (2009) and the current results showed similar profile where increase in initial concentration of naphthenic acid increased the biodegradation rate of trans-4MCHCA. As far as the effect of temperature is concerned, room temperature (20 - 24ºC) was identified as optimum temperature regardless of mode of biodegradation. Under continuous mode of operation (CSTR and biofilm reactors), anaerobic biodegradation was much faster than its aerobic counterpart. For instance the maximum anaerobic removal rate of trans-4MCHCA in the CSTR was 105.4 mg L^{-1} h^{-1}, while the highest removal rate achieved in the aerobic CSTR was 9.6 mg L^{-1} h^{-1}. Similarly, anaerobic biofilm reactor achieved a higher maximum removal rate of 2,028.1 mg L^{-1} h^{-1} compared to a 924.4 mg L^{-1} h^{-1} removal rate in the aerobic biofilm reactor. The overall finding indicated that biodegradation of trans-4MCHCA can be achieved effectively under anaerobic condition with the rates markedly higher than those for aerobic system.

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DEDICATION

This work is primarily dedicated to my auntie, Damawanti

who is the strongest, most optimistic, and most loving woman I know

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NOMENCLATURES AND ABBREVIATIONS

Nomenclature

D – dilution rate (h^{-1})

F –flow rate of the feed ($mL\ h^{-1}$)

HRT – hydraulic residence time (h)

S_i – initial substrate concentration ($mg\ L^{-1}$)

S_r – residual substrate concentration at steady state ($mg\ L^{-1}$)

V – working volume of reactor (mL)

Abbreviations

API – atmospheric pressure ionization

CHWE – Clark hot water extraction

CSS – cyclic steam stimulation

CSTR – continuous stirred tank reactor

ESI – electron spray ionization

FID - flame ionization detection

FT-IR - Fourier transform infrared

GC - gas chromatography

HPLC - high performance liquid chromatography

HRMS - high resolution mass spectrophotometer

IC - ion chromatography

IUPAC - International Union of Pure and Applied Chemistry

LSI-MS - liquid secondary ion mass spectrophotometer

MS - mass spectrophotometry

NAs - Naphthenic acids

OD - optical density

OSPW - oil sand process water

PAHs - polycyclic aromatic hydrocarbons

QTOF - quadruple time of flight

SAGD - steam-assisted drainage gravity

trans-4MCHCA - 4 methyl-1-cyclohexane carboxylic acid

UV - ultraviolet

CHAPTER 1
INTRODUCTION AND RESEARCH OBJECTIVES

Increase in populations of the world and decline in the conventional oil deposits have given the unconventional oil reserves an important role to help meeting the energy demand. Heavy oil, especially oil sands, is an example of unconventional oils. Oil sands are found in about 70 countries around the world with the largest deposits are located in Canada and Venezuela (EMD, 2011). Located in the Northern parts of Alberta and Saskatchewan, Canadian oil sands are considered one of the largest oil reserves in the world (Government of Alberta, 2013). There are three major regions of oil sands in northern Alberta: Athabasca, Cold Lake, and Peace River. Based on the quarterly report from Alberta's Government, approximately there are about 169 billion barrels of oil reserves which underlie 140,200 km^2 of boreal forest, muskeg peat bogs, and northern prairie ecozones (Environment Canada, 2011; Government of Alberta, 2013). These oil sand reserves are recoverable in the form of bitumen, which is extremely viscous heavy oil.

There are two main processes for recoving of the bitumen from oil sand deposits, based on the depth of the bitumen deposit: surface mining and in-situ extraction (CAPP, 2012). These processes required a large volume of water for extraction and separation of the bitumen from oil sands (CAPP, 2012). Almost 80 to 95% of the water used are recycled and the remaining are stored in tailing ponds (Government of Canada, 2011). However, since not all the water could be recycled, these water keep accumulating in the tailing ponds and are referred to oil sand process water (OSPW) (OSRIN, 2010). The OSPW contains toxic organic compounds, heavy metal (including thirteen priority pollutants), and fine clay sediments (Kato *et al.*, 2001; Kelly *et al.*, 2010). Several reports suggested that the primary toxicity of OSPW originates from the presence

of organic carboxylic acid compounds known as naphthenic acids (NAs) (MacKinnon and Boerger, 1986; Rogers *et al.*, 2002; Allen, 2008). Currently, concentration of NAs present in tailing ponds is estimated in the range of 40 to 120 mg L^{-1} (Schramm *et al.*, 2000; Huang, 2011). The discovery of NAs in OSPW has led to a rapid increase in the number of research aiming to assess the structure, toxicity, and impact of NAs on the environment (Grewer *et al.*, 2010). Due to the toxicity of OSPW and the zero discharge policy of Government of Alberta, OSPW is currently stored in tailings ponds for further treatment (Dominski, 2007). Tailing ponds are slowly growing, and to date, they cover more than 170 square kilometers of lands in Alberta (Government of Alberta, 2013).

Many research works have focused to find solutions for treating the tailings ponds water, especially to decrease the toxicity of NAs. Several treatments, including photocatalysis, ozonation, adsorption, and bioremediation were found to be effective in treatment of naphthenic acids (Herman *et al.*, 1994; Quagraine *et al.*, 2005; Scott *et al.*, 2008; Martin *et al.*, 2010; Mishra *et al.*, 2010; Gamal El-Din *et al.*, 2011; Huang *et al.*, 2012). Biodegradation has advantages of being the most environmentally friendly treatment since it removes the toxic pollutants at the low cost and without the extensive use of chemicals. NAs were found to be susceptible to natural degradation by microorganisms (Herman *et al.*, 1994; Holowenko *et al.*, 2002). However, most of the studies of biodegradation of NAs were done in aerobic conditions. Aerobic biodegradation of model naphthenic acids (octanoic acid, trans-4-methyl-1-cyclohexane carboxylic acid, and both isomers of trans-4-methyl-1-cyclohexane acetic acid) has been studied in batch system, continuous stirred tank reactor (CSTR), packed-bed biofilm reactor, and circulating packed-bed reactor (Paslawski *et al.*, 2009; Huang *et al.*, 2011; D'souza, 2012). Effective aerobic

biodegradation of naphthenic acids requires intense aeration to promote microbial activity and biodegradation of NAs. Therefore, anaerobic biodegradation, if successful, is an interesting alternative for treatment of naphthenic acids in which the execessive cost associated with aeration could be eliminated. Moreover, it could be used as a potential in-situ treatment of naphthenic acids in anaerobic stabilization ponds.

In this work, anaerobic biodegradation of a surrogate naphthenic acid, trans-4-methyl-1-cyclohexane carboxylic acid (trans-4MCHCA) under denitrifying conditions was investigated. The specific objectives were: 1)- to study the effects of trans-4MCHCA initial concentration and temperature on the biodegradation and denitrification processes, 2)- to examine the continuous anaerobic biodegradation of trans-4MCHCA in CSTR and biofilm reactors. The CSTR system was used to study the effect of trans-4MCHCA loading rate on its biodegradation and denitrification process, while biofilm reactor was utilized to improve the extent of biodegradation and to investigate the effects of both trans-4MCHCA loading rate and trans-4MCHCA concentration on the processes of anaerobic biodegradation and denitrification. The results of anaerobic biodegradation obtained in the batch, CSTR, and biofilm systems were compared with those obtained previously under aerobic conditions (Paslawski, 2008).

The thesis presented here consists of 5 chapters including introduction, literature review and research objectives, materials and methods, results and discussion, conclusions and recommendations.

CHAPTER 2
LITERATURE REVIEW

2.1. Processing of Oil Sands and Environmental Impacts

Bitumen contained in unconventional oil sands has characteristics as heavy or highly viscous oil, which makes it difficult to extract (GAC, 2007; Government of Alberta, 2013). Bitumen is recovered through two main processes, depending on the location where bitumen was deposited: surface mining and in-situ extraction (CAPP, 2012). Canadian Association of Petroleum Producers (CAPP) reported that 20% of the oil sands reserves were available for surface mining. Bitumen from surface mined oil sands was extracted using Clark hot water extraction (CHWE) process (Han *et al.*, 2009), a method which combines hot water, steam, and caustic addition (eg. NaOH) to separate bitumen from oil sands (Clark, 1939). The rest of 80% of the reserves were located below 70 metres (200 feet), which required drilling methods and wells to recover of these reserves, due to its depth (CAPP, 2012). Drilling methods utilize advanced technology such as steam injection, combustion, or other sources of heat to melt the bitumen. The example of commonly used in-situ methods are steam-assisted drainage gravity (SAGD) (Suncor Energy, 2013) and cyclic steam stimulation (CSS) (Imperial Oil, 2013).

The separation process of bitumen from surface mining oil sands is reported to be much more expensive than extracting conventional oil by drilling (Painter *et al.*, 2010). The recovery process of bitumen (CHWE) in surface mining usually uses hot or warm water and caustic to reduce bitumen viscosity (Schramm *et al.*, 2000; Allen, 2008; Painter *et al*., 2010). The separation process is done by aeration (flotation) to form an oil-containing froth, which later the froth will be skimmed off the surface (Schramm *et al.*, 2000; Painter *et al.*, 2010).

Based on Government of Canada's report, both surface mining and in-situ method extraction of oil sands requires a large volume of water during the process of separation. For example, oil sands surface mining requires three to four barrels of water for producing one barrel of bitumen, while in-situ production required one barrel of new water (Government of Canada, 2011). These production water are stored in tailing ponds for recycling to reduce the amount of fresh water need by about 85% (Government of Alberta, 2011). However, the extraction process still requires fresh water to make up the remaining 15%, therefore more and more water is accumulated in the tailing ponds. Tailings management practices over the last four decades have resulted in large inventories of tailing water, causing problems in land use and water quality because tailing ponds waters can not be returned to the river due to the poor quality of these waters and presence of toxic compounds (Government of Canada, 2011). Currently, there are more than 170 square kilometers of tailings ponds in the oil sands region (OSRIN, 2010; Government of Alberta, 2011). Tailing pond is an area filled with the left over sand, clay, water, fine silts, and other materials that are formed during extraction process (Government of Alberta, 2011). Tailing ponds contain minerals, trace metals and organic compounds that are toxic to organisms (Holowenko *et al.*, 2002; Mahdavi *et al.*, 2012). The toxicity source of tailing ponds is attributed to the presence of a group of organic compound referred to as Naphthenic Acids (NAs) (Allen, 2008). Thus, government of Alberta issued a zero discharge policy to monitor the tailing ponds so that there is no seepage and impact to surface water.

2.2. Naphthenic Acids

The presence of natural carboxylate surfactants was discovered after several analysis of bitumen slurry froth from the oil sand separation process was conducted. Later, these natural carboxylate surfactants were recognized as lower molecular weight NAs (Schramm *et al.*, 1984; MacKinnon and Boerger, 1986). Naphthenic Acids (NAs), based on The International Union of Pure and Applied Chemistry (IUPAC), are defined as "acids, chiefly monocarboxylic derived from naphthenes", while naphthenes are defined as "cycloalkanes especially cyclopentane, cyclohexane, and their alkyl derivatives" (McNaught and Wilkinson, 1997). Naphthenic acids are identified as weak acids with degree of proton dissociation constant ranging from 10^{-5} to 10^{-6} (pKa's value approximately between 5 to 6), and their solubility being dependent on the pH of surrounding medium (Clemente and Fedorak, 2005; Toor *et al.*, 2013). It was suggested that, based on their pKa value, NAs are negatively charged at neutral and alkaline pH conditions (Allen, 2008; Liang *et al.*, 2011).

NAs have chemical characteristic similar to carboxylic acids. NAs are stronger acids compare to higher fatty acids, but slighly weaker compare to lower molecular carboxylic acids, such as acetic acid (Brient *et al.*, 1995). In petroleum and petrochemical industries, NAs are known as naturally occuring compounds present in crude oil. NAs are also found in ground and surface waters as contaminant due to run off water/groundwater flushing from reclamation process of tailings or seepage water from different mature fine tailing which has direct contact with river water (Schramm *et al.*, 2000; Amstrong *et al.*, 2010; Grewer *et al.*, 2010). NAs mixtures which are released from bitumen extraction process have concentrations ranging from 40 to 120 mg L^{-1} (Holowenko *et al.*, 2002; Clemente *et al.*, 2003). During the extraction process, NAs react with

sodium hydroxide and form a carboxylate. This is why, in the oil sand process water (OSPW), NAs are found in the form of sodium naphthenates (Toor, 2012). In general, NAs are well-known as non-volatile, chemically stable, toxic compounds, that do not dissolve or slightly dissolve in water (< 50 mg L^{-1}). NAs are soluble in organic solvents and oils due to their high pKa value (Brient *et al.*, 1995; Huang, 2011). NAs have boiling points ranged from 250 to 350°C. Under normal temperature and condition, NAs appear to be stable (D'souza, 2012; Toor, 2012). NAs have a strong odour which is caused by the presence of phenolic and sulphur impurities in the NAs' mixture. NAs have physical appearance of being either clear or brown viscous liquids. NAs are commonly used as fuel additives, wood preservatives, paint driers, aluminium ceramics, anti-wear lubricants, and in the process of manufacturing tires (Brient *et al.*, 1995; Paslawski *et al.*, 2009, Huang *et al.*, 2012). In wood preservation industries, NAs are utilized through reaction with different metals to form metal naphthenate salts such as copper and zinc naphthenate (Niemi *et al.*, 1998). Copper and zinc naphthenate usually serve as fungicide to control fungal rot, mildew, wood decay, and wood-boring insects, thus they are suitable to preserve material and woods (Health Canada, 2010).

Many studies were conducted using different analytical techniques to study composition and structure of NAs. Generally, NAs are defined as compounds that have the chemical formula of $C_nH_{2n+z}O_2$, where n is the number of carbon atoms and z is number of hydrogen atoms missing in the cyclic NAs. NAs which are present in the oil sands process waters consist of compounds with n values ranging from 7 to 30, while the z value are in the range 0 to -12 (Del Rio *et al.*, 2006; D'souza, 2012). However, this definition was referred to classical naphthenic acid (Grewer *et al.*, 2010). Herman et al. (1994) classified NAs into three categories of components based on their

structures: one or more five- or six- carbon cycloalkane rings, an aliphatic side chain of various lengths, and a carboxylic acid group. Later, using both mass spectrometry and GC-MS, the presence of aromatic naphthenic acids in crude oils and OSPW were detected (Hsu *et al.*, 2000; Jones *et al.*, 2012). Aside from aromatic compounds, more complex molecules such as multiple carboxyl groups, phenolic groups, oxy-NAs ($C_nH_{2n+z}O_x$: compounds with x= 3 to 5), as well as heteroatoms such as N and S were also detected as part of NAs structure (Headley *et al.*, 2009; Grewer *et al.*, 2010; Gamal El-Din *et al.*, 2011; Huang *et al.*, 2012). Based on this discovery, the classical definition of NAs of being cycloalkane rings was inadequate. Recently, West et al. (2013) reported the possibility of the presence of isobaric 'SO_2' species of NAs that might have been ignored because of inconsistent masses accuracy and/or derivatisation reaction. The classical structures of NAs are shown in Figure 2.1.

Naphthenic acids are known to be toxic to aquatic biotas and mammals (Rogers *et al.*, 2002; Gosselin *et al.*, 2010). The toxicity of NAs was believed to be caused by its surfactant property (Rogers *et al.*, 2002). Surfactants are chemicals that can lower the surface tension of a liquid and act like a detergent. The surfactant characteristic gives possibility for NAs to penetrate the cell wall of living organisms and cause membrane disruption (Schramm *et al.*, 2000). Subchronic test of NAs in rats showed that NAs had ability to damage brain, hearth, muscle, liver, and blood (Rogers *et al.*, 2002).

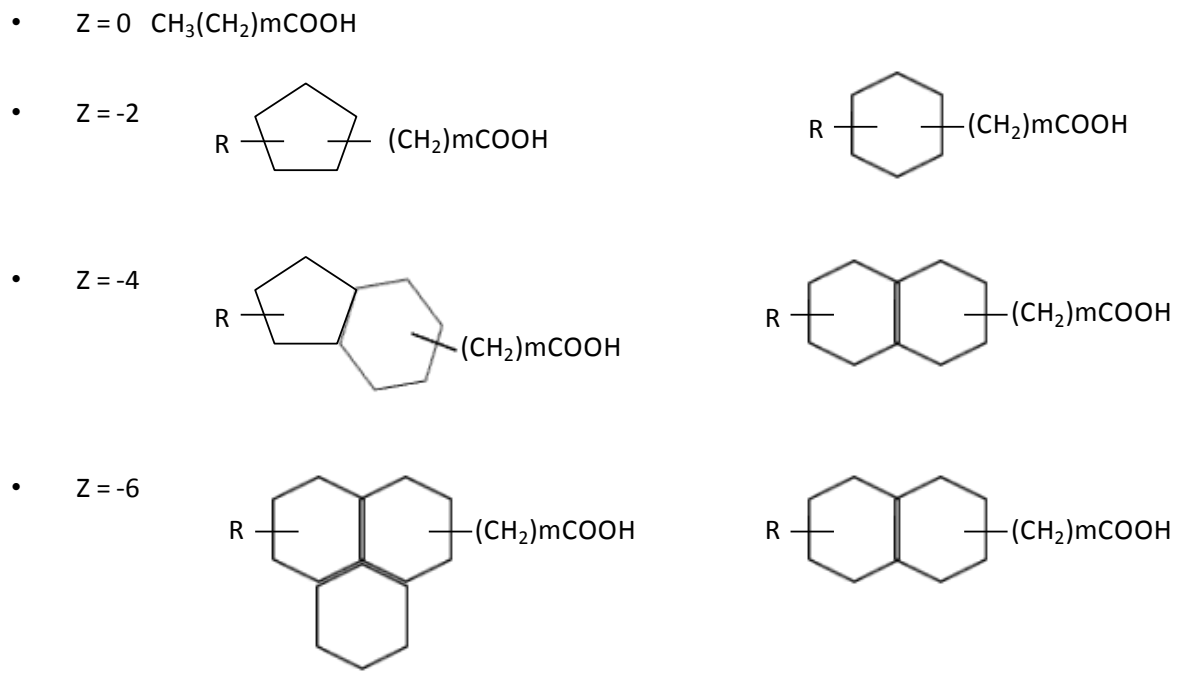


Figure 2.1. Structures of classical naphthenic acids, where z represents hydrogen atoms missing due to the cyclic structure, R is an alkyl chain, and m is the number of CH_2 units (Hao *et al.*, 2005)

NAs have been proposed to have toxicity effect to aquatic biotas through general narcosis step, leading to membrane disruption and osmotic homeostasis disruption (Quagraine *et al.*, 2005b; Frank *et al.*, 2008; Toor, 2012). Using mouse as the subject, Garcia-Garcia et al. (2011) showed that NAs in OSPW caused immunotoxic effect that may impair the function of antibody on exposed host to defend against infectious disease. NAs are also shown to have a lethal effect to freshwater fish species, and non-lethal effect causing changes in gill and liver followed by decrease in blood glucose levels and leukocyte counts, and increased in muscle glycogen (Rogers *et al.*, 2002; Kannel and Gan, 2012). In mice embryonic development stage, exposure of Mildred Lake settling basin surface water and commercial sodium naphthenate causes spinal deformities,

decrease in number of eye pigmentations, little or missing tail, and early mortality (Peters *et al.*, 2007).

It has been reported that lower molecular weight of NAs had higher toxicity value compared to NAs with higher molecular weight; also that NAs with lower number of cycloaliphatic rings were more toxic than the one which contained higher number of cycloaliphatic rings (Holowenko *et al.*, 2002; Frank *et al.*, 2008; Huang, 2011). Many studies showed that lower molecular weight of NAs are more available in nature (Headley and McMartin, 2004; Scott *et al.*, 2005). Nonetheless, the lower molecular weight of NAs are more susceptible to natural biodegradation, therefore over extended period of time, toxicity levels in the tailing ponds was observed to decrease with aging (Holowenko *et al.*, 2002; Huang, 2011; D'souza, 2012).

In addition to its toxicity, NAs are also known to be the major cause of corrosion in oil industries due to their acidic characteristic (Allen, 2008; Huang, 2011). It was reported that at temperature between 220°C and 400°C in distillation units, with the NAs' corrosion peak at temperature of 320°C, could cause equipment failures (Schramm *et al.*, 2000; Barrow *et al.*, 2003; Quagraine *et al.*, 2005; Sixian *et al.*, 2013). At condition above 400°C, decomposition of NAs appeared to happen, thus high temperature eliminated the presence of NAs; meanwhile at temperature below 220°C, the corrosion reaction was inhibited because there was a high activation energy barrier inhibiting NAs (Sixian *et al.*, 2013). The reactions causing corrosion by NAs is shown to be endothermal reactions (Sixian *et al.*, 2013).

2.3. Identification of Naphthenic Acids

Many attempts were done to analyze and isolate individual NAs compounds from the oil sand especially, where the first attempt to characterize NAs components were dated back before year of 1955 (Lochte and Littmann, 1955). Various methods have been developed to analyze and characterize NAs compounds. The methods which are reported to be succesfully detecting NAs are gas chromatography with flame ionization detector (GC-FID) (Paslawski, 2008; D'souza, 2012), fast atom bombardment mass spectrometry (FABMS) (Fan, 1991), electronspray ionization mass spectrometry (ESI-MS) (Martin *et al.*, 2008), combination of GC-MS (Scott *et al.*, 2008), Fourier transformed-infrared (FTIR) spectroscopy (MacKinnon and Boerger, 1986; Herman *et al.*, 1994; Grewer *et al.*, 2010), high performance liquid chromatography (HPLC) (Scott *et al.*, 2005), combination of HPLC and quadrupole time-of-flight mass spectrometer (QTOF-MS) (Bataineh *et al.*, 2006), or any combination of two or more methods.

Gas chromatography/mass spectrometry is a method that offers ability to resolve the constituent organic compounds in the mixture, however this method is only able to provide relative quantities instead of absolute concentration (Holowenko *et al.*, 2002; Nodwell, 2011). Fourier-transform infrared spectrometry (FTIR) is a method that is commonly used in industries to determine bulk concentration of NAs in oil sands process water. However, FTIR might be giving artificial high readings due to naturally occuring, non-toxic organic acids in the process water (Scott *et al.*, 2008; Nodwell, 2011). HPLC, on the other hand, gives the accurate total concentration of NAs. However, HPLC has disadvantage of not being able to differentiate the various organic compounds in the mixture like the GC/MS method (Yen *et al.*, 2004; Nodwell,

2011). Due to the complexity of NAs mixture, complete identification of NAs, either individual compounds or as mixtures, were difficult to achieve (Quagraine, 2005; Huang, 2011).

2.4. Potential Methods for Treatments of Naphthenic Acids in Oil Sand Process Water

Many problems are detected from the presence of NAs in process water and tailing ponds. Some of the problems that can be mentioned are corrosion of process equipment, calcium naphthenate precipitation, production of highly viscous emulsions (which behave like sludge) formed by salts of aliphatic organic acids which could accumulates at separation vessels, and acidity (Brient *et al.*, 1995; Hanneseth *et al.*, 2010). These problems are caused mainly by the characteristic of tailings ponds water which are alkaline, slightly brackish (MacKinnon and Sethi, 1993), and somewhat toxic to mammals, but extremely toxic to aquatic biota (Brient *et al.*, 1995; Rogers *et al.*, 2002). The alkaline characteristic of tailing ponds water is a result of the use of sodium hydroxide in the extraction process. There are several options for treating NAs to reduce its toxicity, recycling of water, as well as restoring sustainable aquatic ecosystems (Allen, 2008). These include: advanced oxidation (photocatalysis) (Mishra *et al.*, 2010), ozonation (Scott *et al.*, 2008; Martin *et al.*, 2010), adsorption (Janfada *et al.*, 2006; Gamal El-Din *et al.*, 2011), and bioremediation (Herman *et al.*, 1994; Quagraine *et al.*, 2005; Huang *et al.*, 2012; D'souza, 2012).

2.4.1. Advanced Oxidation (Photocatalysis)

Photocatalysis is an acceleration process of photoreaction (using either UV or visible light) in the presence of a catalyst which could be used to treat wastewaters and gaseous pollutants (Chen, 1997). Photocatalysis is established from a redox (reduction oxidation) reaction which takes place to form electron-hole pairs and generates free radical for secondary reactions (Linsebigler

et al., 1995). Illumination of an organic compound (e.g. aromatic, organochloride, and organophosphorus) by UV/visible light in the presence of a catalyst will oxidize and mineralize organic compounds at catalyst's surface (Chen, 1997). The process starts by production of radicals at the surface, dissolution of radicals, and reaction with organic compounds (Chen, 1997). A research group studied photocatalysis of NAs, using growth chamber with full spectrum artificial solar radiation, and reported that NAs were not able to absorb light in the solar wavelength region, thus direct photolysis from the sun is not possible (McMartin *et al.*, 2004). Then, several groups reported that UV_{254} was the most effective source of radiation for photolysis of NAs (McMartin *et al.*, 2004; Afzal *et al.*, 2012). Based on these findings, further study on photocatalysis was conducted. The result showed that fluorescent lamp with UV_{254} in the presence of titanium dioxide (TiO_2) as catalyst could effectively degrade fluka NAs and OSPW NAs (Mishra *et al.*, 2010). TiO_2 was chosen because, as a catalyst, it was effective, inexpensive, non-toxic, chemically stable, and was not affected by photo corrosion (Doll and Frimmel, 2005, Mishra *et al.*, 2010). Mishra et al. (2010) reported that UV_{254} fluorescent lamps degraded NAs with higher z values (multiple rings) more rapidly than the linear or single ring NAs.

Combination of UV light and hydrogen peroxide (H_2O_2) was evaluated for possible treatment of OSPW (Horowitz *et al.*, 1996; Afzal *et al.*, 2012). The results showed that UV/H_2O_2 effectively treated NAs compounds, especially those that fall into group of cyclohexanoic acid. The effect results also showed that NAs compounds which contained more rings experienced slower degradation than the one with less rings (Afzal *et al.*, 2012). In conclusion, photolysis, using UV_{254}, appears as an effective process to cleave higher molecular weight NAs into smaller

fragments, which is easier for microorganisms to degrade (Kannel and Gan, 2012), thus photolysis can be used as pre-treatment of naphthenic acids prior to biological treatment.

Study on effect of pH on photocatalysis of NAs is also important. This due to the fact that optimum condition of bitumen liberation is at a pH around 11.3, which is an alkaline condition (SrinivasaRajagopalan, 2010). A group of researchers compared advance oxidation mineralization process of NAs, using four different catalysts including TiO_2, IO_4^-, $S_2O_8^{2-}$, and H_2O_2, under different pH conditions ranged from 8 to 10 (Liang *et al.*, 2011). The result showed that in the absence of UV light, degradation of NAs did not occur because the four catalysts which were used could not directly oxidize NAs without radical species (Liang *et al.*, 2011). Radical species is formed when the catalysts are illuminated with UV light, which is the accelerator in photocatalysis reaction (Liang *et al.*, 2011). The same group reported that under high pH value, TiO_2 catalyst had a poor performance because high pH caused potential confounding effects such as involvement of other radicals or the TiO_2 surface (Liang *et al.*, 2011). As it was previously mentioned, oil sand extraction process water has an alkaline pH, which could deactivate TiO_2 catalyst and might not be able to mineralize NAs from extraction process waters.

Photocatalysis, using microwave radiation in the presence of TiO_2, was able to degrade fluka NAs and extracted OSPW NAs (Mishra *et al.*, 2010). The microwave technology was introduced by Kong et al. (2006) for the first time to remove NAs from diesel oil. Using the same technology, Mishra et al. (2010) studied the degradation of NAs from water and explained how microwave system worked by alternating electromagnetic wave, resulting in polarization of NAs

molecules and denaturation of the compounds which occurred through the breaking of carbon-carbon bonds. The microwave treatment was shown to be effective to reduce toxicity of NAs at short period of time (Mishra *et al.*, 2010). Similarly, photocatalysis using microwave treatment prefers higher molecular weight to lower molecular weight of NAs; therefore photocatalysis can be used as a potential pre-treatment of NAs prior to biological treatment.

2.4.2. Ozonation

Ozonation process works through self-decomposition of ozone to oxygen and radical oxygen, which lead to formation of hydroxyl radical. The radicals, which was produced from the self-decomposition reaction, could be used to degrade organic compounds. Ozonation study was conducted originally to evaluate the effectiveness of the method to remove NAs from OSPW and reduce its toxicity level in addition to biodegradation (Scott *et al.*, 2008). Ozone was reported to have standard reduction potential value of 2.07 V, which was considered to be a powerful oxidant available in water treatment process (Fu *et al.*, 2008). Ozonation process was capable of removing 95% of NAs at pH of 8 to 8.2, but there was no corresponding decrease in total organic carbon (TOC), suggesting incomplete NAs oxidation happened under this condition (Liang *et al.*, 2011). In addition to the pH, oxidation of NAs using ozonation process was affected by the presence of radical species, such as hydroxyl radical ($OH^{\bullet}$) (Scott *et al.*, 2008; Liang *et al.*, 2011). Hydroxyl radical is believed to be a rapid and indiscriminate oxidant, but hydroxyl radical will react with NAs in ozonation reaction only at elevated pH (Buxton *et al.*, 1988; Liang *et al.*, 2011). Scott et al. (2008) showed the advantage of ozone treatment for reducing the color of OSPW and increasing the value of IC_{20}. IC_{20} value is used to determine the level of water toxicity; with the higher numbers representing lower toxicity of water. Microtox assay showed

that in order to obtain non-toxic treated OSPW complete destruction of NAs was not necessary (Scott *et al.*, 2008). The focus of most studies was therefore, to reduce toxicity of NAs through degradation of NAs compounds with lower number of rings (Scott *et al.*, 2008).

Ozonation worked best when it was combined with biodegradation or adsorption as a pre-treatment step (Martin *et al.*, 2010; Gamal El-Din *et al.*, 2011), because ozonation was shown to have ability to break down highly branched isomers to the more linear forms (it had preference of oxidizing NAs with more alkyl branching), the which the most recent advance technology is not able to perform (Martin *et al.*, 2010). Molecular structures of NAs influenced the degradation rate of NAs by ozonation. NAs compounds with higher z numbers (containing more rings) were degraded faster than those with lower z numbers (Mohamed *et al.*, 2008; Afzal *et al.*, 2012). This finding shows that ozonation has the opposite effect to photocatalysis (UV/H_2O_2) treatment. However, OSPW waters which are treated by ozonation appeared to have higher toxicity effect compared to the one with no treatment (Garcia-Garcia *et al.*, 2011). This might be caused by greater concentration of lower molecular weight of NAs after the treatment. As previously mentioned, lower molecular weight of NAs have a higher toxicity value compare to the one with higher molecular weight. Therefore, the treated water, when it was exposed to mammals, could alter the liver gene expression, which affected mammals' immune system, causing cell inflammation (Garcia-Garcia *et al.*, 2011).

2.4.3. Adsorption

Adsorption is the attachment of atoms, ions, or molecules, in the form of gas, liquid, or dissolved solid on to a solid surface (adsorbent). Adsorption method had reported to effectively removed

recalcitrant organic compounds from aqueous solution (Zoschke *et al.*, 2011; Zubot *et al.*, 2012), for example, activated petroleum coke that was produced from physical or chemical activation showed a tendency to remove polycyclic aromatic hydrocarbons (PAHs), organic contaminants presence in tailing ponds waters (Yuan *et al.*, 2010). Petroleum coke could be activated using potassium hydroxide (KOH) in short period of time to produce higher surface area of products to treat PAHs (Di Panfilo and Egiebor, 1996; Small *et al.*, 2012).

Petroleum coke is a "carbonization product of high-boiling hydrocarbon fractions obtained in petroleum processing", that can be a potential viable adsorbent to treat OSPW (particularly total acid-extractable organics and NAs) so that the water can be returned to the environment and/or for certain plant reuse application (Nic *et al.*, 2012; Zubot *et al.*, 2012). The work done by Zubot et al. (2012) showed that activated petroleum coke was able to adsorb NAs, specially NAs with higher molecular weight and higher ring structures. Peng et al. (2002) reported that adsorption of NAs were unaffected by the geometric structures of NAs, as the value of adsorption coefficient (K_d) and adsorption energy of cis- or trans- isomers were observed to be similar. Addition of inorganic salt (e.g. calcium chloride) might affect the adsorption rate through the change in pH, which could change the K_d value of adsorbant (Peng *et al.*, 2002; Smith *et al.*, 2008). The studies showed that the removal of organic compounds (including NAs) by petroleum coke adsorption followed a pseudo-second order kinetic model (Yuan *et al.*, 2010; Zubot *et al.*, 2012). Due to its high level sulphur content which can limit petroleum coke's performance if used at high temperature, petroleum coke is not used as a solid fuel; thus the use of petroleum coke as adsorbant is ideal due to the low cost of adsorbant and its effectiveness (Yuan *et al.*, 2010; Zubot *et al.*, 2012).

2.4.4. Biodegradation

Biodegradation is the process of decomposition of organic compounds by various organisms (including algae, plant, and bacteria). The process starts by adaptation of organisms to utilization of pollutant (especially in case of recalcitrant pollutants), release of various enzymes by organisms, and conversion of pollutant to harmless products. Since in biodegradation, living organisms (especially microorganisms) play the role of biocatalyst, the process is highly affected by environmental temperature. Microorganisms, specifically bacteria are categorized into four different types depending on the temperature in which they thrive: 1)- psychrophilic bacteria, those which can live at temperature around -5 to 15°C, 2)- mesophilic bacteria, microorganisms that can live at temperature ranging from 20 to 45ºC, 3)- thermophilic bacteria, that are able to survive under temperature ranging from 50 to 70°C, and the last category, 4)- hyperthermophilic bacteria, which survive at temperatures of 70 to 122°C (Madigan and Martinko, 2005; Takai *et al.*, 2008).

Based on oxygen demand of the bacteria, biodegradation can be carried out aerobically or anaerobically. Aerobic biodegradation is a process where oxygen serves as the terminal electron acceptor. By contrast, anaerobic biodegradation is a process where other compounds such as nitrate, sulphate, etc. serve as the terminal electron acceptor (i.e. oxygen could be toxic to anaerobic microorganisms). A study on crude oil spill showed that both aerobic and anaerobic biodegradations were effective in treatment of the contaminated site (Baedecker *et al.*, 1993). Depends on the treatment approach, biodegradation can be achieved either in-situ or ex-situ. In-situ biodegradation is a process where conversion of contaminants takes place in the polluted site. On the other hand, ex-situ biodegradation or bioremediation refers to a process where the

contaminated media (soil or water) are remediated off site, usually in properly designed treatment system. Overall, biodegradation process shows to follow a sigmoidal curve when its kinetic is plotted. Biodegradation had been reported to successfully treat various organic compounds, including naphthenic acids, polycyclic aromatic carboxylic acid, phenolic compounds, benzene, etc. (Herman *et al.*, 1993; Sinha *et al.*, 2009; Giesy *et al.*, 2010).

2.4.4.1. Phytoremediation

Phytoremediation is a specific form of biodegradation where microorganisms associated rhizosphere contributes to the removal and/or transform of organic contaminants, including naphthenic acids (Susarla *et al.*, 2002, Biryukova *et al.*, 2007). Algae communities have been associated with tailing ponds water especially the Athabasca Oil Sands tailings (Quesnel *et al.*, 2011). Phytoremediation application has demonstrated the importance of rhizosphere microbial communities, where plant-bacterial combinations are able to enhance degradation of contaminants and accelerate light-induced transformation of organic compounds (Susarla *et al.*, 2002; Biryukova *et al.*, 2007; Headley *et al.*, 2008). Some study have shown that diversity in rhizobacterial population of phytoremediation plants are necessary to degrade NAs and minimize their toxicity (Headley and McMartin, 2004; Biryukova *et al.*, 2007).

Removal process of contaminants in phytoremediation happens through three steps: 1)- stimulation of rhizobacterial transformation, 2)- slowing of contaminant transport from the rooting zone as a result of adsorption of increased evapo-transpiration, 3)- and contaminants uptake by plant followed by metabolism, volatilization, or accumulation (Herman *et al.*, 1996; McMartin, 2003; Quagraine *et al.*, 2005; Kannel and Gan, 2012). Phytoremediation is affected

by factors including: physical and chemical properties of the organic compounds (e.g. water solubility, molecular weight, vapor pressure, and octanol-water partition coefficient), environmental characteristics (e.g. temperature, pH, organic matter, and soil moisture content), and plant characteristics (e.g. root types and enzymes types) (Susarla *et al.*, 2002). Phytoremediation process is more efficient in degradation of lower molecular weight NAs over higher molecular weight thus it is effective in reducing the toxicity of NAs (Biryukova *et al.*, 2007). Geometrical structure of NAs also plays an important role in biodegradation rate in phytoremediation, where algae prefers to degrade trans-isomers to cis-isomers due to the open trans-geometry compared to cis-geometry (Headley *et al.*, 2008). However, it was noticed that pH and dissolved organic carbon (DOC) levels did not appear to have a significant effect on phytoremediation rate of NAs (Biryukova *et al.*, 2007).

Unicellular alga *Dunaliella tertiolecta* has been reported to have the ability to degrade five different model naphthenic acids: cyclohexane carboxylic acids, cyclohexane acetic acids, cyclohexane propionic acids, cyclohexane butyric acids, and 1,2,3,4-tetrahydro-2-naphthoic acids (Quesnel *et al.*, 2011). These authors proposed two possible metabolism pathways for cyclohexane carboxylic acid degradation by *Dunaliella tertiolecta* algae: 1)- conversion of cyclohexane carboxylic acid into cyclohexylidene acetic acid through α–oxidation and 2)- conversion of cyclohexane carboxylic acid to 1-cyclohexene acetic acid through biotransformation (Quesnel *et al.*, 2011). Several studies compared the phytoremediation of cyclohexane carboxylic acid and cyclohexane acetic acid and concluded that cyclohexane carboxylic acid is less recalcitrant than cyclohexane acetic acid due to the position of β carbon not being the tertiary carbon (Iwaki *et al.*, 2008; Whitby, 2010; Quesnel *et al.*, 2011). Having a

secondary carbon in the structure allows step-wise β-oxidation to happen, causing the ring opening and further degradation of the resulting fatty acid-like compound by simple respiratory process (Iwaki *et al.*, 2008; Whitby, 2010; Quesnel *et al.*, 2011). Phytoremediation has limitation because the treatment cannot be applied to every contaminated site due to different soil and climate conditions that might not be suitable for the target plant (Susarla *et al.*, 2002).

2.4.4.2. Aerobic Biodegradation

An early study in 1994 reported that toxicity of commercial mixture of NAs was reduced over time by the activity of microorganisms, even though gas chromatography did not show the reduction of NAs' concentration (Herman *et al.*, 1994). Later, Han et al. (2009) observed the reduction of NAs concentration by biodegradation process from ten different sites of settling basins where the older OSPW contained less NAs than the active settling basin. It was also observed that the degradation of NAs correlated by decrease in quantity of dissolved organic carbon (DOC) and respired CO_2 (Videla *et al.*, 2009). The commercially available NAs were found to be degraded easily compared to the one extracted from tailings ponds (Scott *et al.*, 2008). Chemical structure of NAs have been shown to affect the biodegradation process, where the lower molecular weight NAs were preferentially degraded over the higher molecular weight NAs, and the one that contain multiple branched alkyl chains and methyl substituted cycloalkane rings were more recalcitrant compare to the one that didn't have alkyl branch (Holowenko *et al.*, 2002; Paslawski *et al.*, 2009; Johnson *et al.*, 2011; Huang *et al.*, 2012). As previously mentioned, cycloalkanes NAs were reported to be more recalcitrant to biodegradation process compared to n-alkanes and aromatics due to their molecular structures (Iwaki *et al.*, 2008). Some studies have reported that intramolecular hydrogen bonding also influences biodegradation rate. For example,

in geometric NA isomers, trans-isomer degraded faster than cis-isomer since cis-geometric had a closed structure, thus it was less stable than the open geometry of trans-geometry (Headley *et al.*, 2002; Huang *et al.*, 2012). It is important to note that biodegradation studies using commercial NAs alone do not accurately reflect the biodegradation process of NAs in OSPW due to many factors that influenced biodegradation rate (Toor, 2012).

Using radiolabeled NAs that represented different z classes, Lai et al. (1996) reported that addition of phosphate increased the NAs' biodegradation rate. Additionally, decrease in temperature reduced the biodegradation rate of NAs (Paslawski, 2008), showing that environmental factors also influenced the biodegradation rate. Recently, a research group tried to study simulated aerobic wetland, mimicking OSPW containing ponds, as an alternative biodegradation treatment for NAs (reclamation strategy) and suggested that the observed biodegradation of NAs were primarily influenced by the amount of oxygen present and type of indigenous microorganisms living in both OSPW and wetland sediment (Toor *et al.*, 2013). Toor et al. (2013) reported that initially, the simulated wetland treatment had a high rate of sorption of NAs on the first week of the treatment. However, this wetland treatment had long hydraulic residence time (HRT) which caused saturation on binding sites (sediment that acts as adsorbents). The saturated adsorbents and sufficient available oxygen in simulated wetland stimulated biodegradation process, taking over the sorption process (Toor *et al.*, 2013). Also, it was reported that NAs had a low estimated adsorption coefficient (K_d) which caused NAs to stay in their highly soluble, dissociated form, thus adsorption of NAs by sediment was unlikely (Schramm *et al.*, 2000; Toor, 2012). There is a promise in utilizing radio-labelled NAs and stable

isotopes in bacterial degradation to discover the mechanisms involved in biodegradation (Videla *et al.,* 2009; Toor, 2012).

Origin and composition of microbial culture is another factor that affected the rate of biodegradation. A mixed culture of microorganisms was shown to degrade NAs better than a single species culture, indicating that there was a unique metabolic interaction between different microorganisms (Del Rio *et al.,* 2006). Degradation of recalcitrant NAs (specifically cycloalkane rings with methyl alkyls) required mixed cultures (Herman *et al.,* 1993; Headley *et al.,* 2002; Johnson *et al.,* 2011). Most of the biodegradation studies were successfully done in aerobic condition (Clemente *et al.*, 2004; Han *et al.*, 2008), while in the anoxic condition it was reported that there was no or little biodegradation (Han *et al.*, 2009). Different strains such as *Pseudomonas putida* (Del Rio *et al.*, 2006; Johnson *et al.*, 2011), *Pseudomonas fluorescens* (Del Rio *et al.*, 2006), *Acinetobacter anitratum, Alcaligenes faecalis* (Blakley and Papish, 1982; Johnson *et al.*, 2011), and Mycobacterium genus (Johnson *et al.*, 2012) have been identified as microorganisms that successfully degraded NAs (both aliphatic and aromatic) under aerobic condition.

2.4.4.3 Pathway Proposed for Aerobic Biodegradation of NAs and their Microbial Biotransformation

Process of complete degradation of organic compounds into inorganic end products by microorganisms is referred to as mineralization (Suthersan, 1999). In general, mineralization process is always accompanied by the growth of microorganism, utilization of O_2, production of CO_2, conversion of toxic compounds to innocuous organic compounds, and production of water (Herman *et al.,* 1994). Herman et al. (1994) utilized cyclohexane pentanoic acid (CHP) to study

mineralization of NAs and reported that degradation reaction started from oxidation of carboxylated aliphatic side chain to oxidation of cycloalkane ring. There are several mechanisms proposed for the biodegradation of aliphatic and alicyclic carboxylic acids, for example: β-oxidation, combination of α- and β- oxidation, and aromatization pathways (Blakley, 1978; Han *et al.*, 2008). By definition, β-oxidation is a process by which fatty acid molecules are broken down to generate acetyl CoA. Beta oxidation removes two carbon atoms at the end of acyl-CoA giving acetyl-CoA as the product, that can also be broken down into carbon dioxide. Alpha oxidation occurs when there is an alkyl group present at the beta carbon of fatty acyl-CoA that blocks beta oxidation. In short, alpha oxidation removes one carbon atom from the carboxyl end, giving formyl-CoA as the product that could be further broken down into carbon dioxide. Biodegradation of highly alkyl-branched compounds are difficult to proceed due to more complex pathways that require the combination of alpha and beta oxidation; microorganisms that are able to utilize cyclohexlacetic acid by alpha oxidation are also not widely distributed (Rontani and Bonin, 1992; Han *et al.*, 2008).

Blakley (1978) reported that biodegradation of cyclohexane carboxylic acid followed β-oxidation pathway involving aromatization process through ortho or meta cleavage, giving coenzyme A and aromatic intermediates. These intermediate compounds are the result of biotransformation by microorganisms. In reviews of cycloalkanes biodegradation, Trudgill (1984) and Perry (1984) concluded that microorganisms followed a predominant β-oxidation pathway for degradation of carboxylated cycloalkenes, indicating that microorganisms which are able to oxidize aliphatic side chains could also be able to degrade cycloalkane rings. The proposed biodegradation pathway of cyclohexane carboxylic acid is shown in Figure 2.2. A recent study reported that

NAs compounds could affect utilization of different electron acceptors in various ways. For example, when adding NAs into ammonia containing solution, NAs did not affect ammonia removal process by mixed culture of nitrifying bacteria (Misiti *et al.*, 2013). In fact, NAs were degraded at the same time as ammonia (under aerobic condition), even though NAs were not degraded strictly by nitrifying bacteria (Misiti *et al.*, 2013).

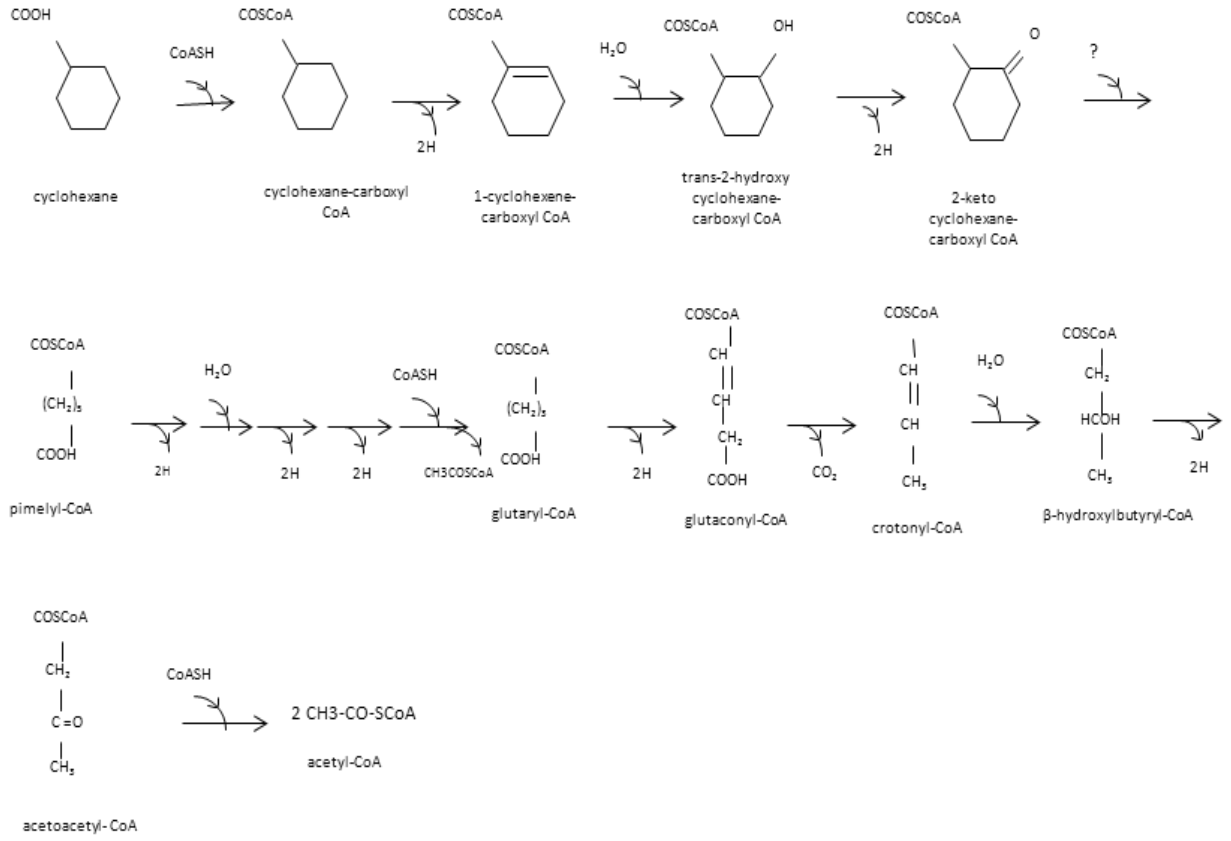


Figure 2.2. The proposed biodegradation pathway of cyclohexane carboxylic acid (Blakley, 1978)

2.4.4.4. Anaerobic Biodegradation

Biodegradation studies in anaerobic condition (in the absence of oxygen) had been done as an alternative to aerobic biodegradation. It is important to better understand the process of anaerobic biodegradation, especially for biodegradation treatment at the lower aquifers zone, where aeration is absent and anaerobic conditions prevail.

Since oxygen is absent in anaerobic condition, biodegradation required other electron acceptors, such as: nitrate, nitrite, and sulfate, for oxidation the organic compounds. Many bacterial species were able to degrade aromatic rings under anaerobic condition using electron acceptors such as: nitrate, sulfate, ferric iron, manganese or carbon dioxide (Evans, 1977; Essaid *et al.*, 1995; Widdel and Rabus, 2001). The use of sulfate as electron acceptor have contributed in mineralizing organic carbons (Muyzer and Stams, 2008). Anaerobic biodegradation with sulfate is more advantagous compared to oxygen because sulfate is not consumed by abiotic reactions with ferrous iron or sulfide, thus it did not form iron oxide precipitates that could cause plugging in *in-situ* treatment (Anderson and Lovley, 2000; Foght, 2008). However, using sulfate as terminal electron acceptor is not favorable due to ability of sulfate-reducing bacteria to reduce sulfate to sulfide, which is highly reactive, corrosive, and toxic (Muyzer and Stams, 2008). The anaerobic biodegradation of aromatic and aliphatic compounds, expecially cyclohexane, under anoxic condition is well-studied (Foght, 2008; Musat *et al.*, 2010). However, the process is not seen as favorable as the aerobic process since it is always believed that anaerobic biodegradation has slower rate compared to aerobic biodegradation. Aerobic microorganisms are also believed to have the ability of degrading wider range of hydrocarbon compounds than anaerobic microorganisms. There is an argument in support to anaerobic bioremediation, where anaerobic

remediation were reported to degrade aromatic hydrocarbons that were the most water-soluble such as: benzene, toluente, xylenes, and ethylbenzenes (Widdel and Rabus, 2001).

Comparison of both the aerobic and anaerobic processes on a study of simulation of biodegradation at crude oil spill site was reported (Essaid *et al.*, 1995). The simulation site was set in a way where the top boundary of aquifer was aerated with constant rate, while the bottom part had no flow to allow prevalence of anaerobic conditions. The result showed that anaerobic processes accounted for 60% of degradation, while aerobic degradation accounted for about 40% of total degradation (Essaid *et al.*, 1995). This finding opened up possibility to treat contaminated site by in-situ methods. At the same time, petroleum hydrocarbons were reported to be susceptible to anaerobic degradation by sulfate-reducing and nitrate-reducing bacteria (Fukui *et al.*, 1999). Similar to the crude oil spill case, anaerobic biodegradation of petroleum hydrocarbons, especially alkylbenzenes and n-alkanes, occurred simultaneously with reduction of sulfate to sulfide. Later, successful biodegradation of several different aromatic hydrocarbons in the presence of different electron acceptors, such as: nitrate, sulfate, carbon dioxide, iron (III), and chlorate, was reported in another study (Foght, 2008). Using benzene as example of aromatic compounds, several groups reported under denitrifying condition, aromatic biodegradation had lower microorganisms yield compared to the theoretical value, suggesting that aromatic biodegradation was an enzyme site-specific and unpredictable (Ulrich and Edwards, 2003; Foght, 2008). Boll et al. (2002) proposed the oxidation mechanisms of aromatic and hydrocarbon in the absence of oxygen, using bacterial enzymes, through two reactions: 1)- reduction of aromatic ring of benzoyl-coenzyme A and 2)- addition of fumarate to hydrocarbons. These two reactions converted the aromatic compounds and hydrocarbons into products that could be

further oxidized through beta-oxidation (Boll *et al.*, 2002). In the case of aromatic compounds and hydrocarbons, it is believed that these compounds are recalcitrant to anaerobic biodegradation due to two reasons: 1)- the resonance energy of the aromatic ring; 2)- the inertness of C-H and C-C bonds in hydrocarbons (Boll *et al.*, 2002).

Several studies reported the successful biodegradation of gasoline- and diesel-contaminated groundwater, which contained toxic organic compounds such as monoaromatic compound (benzene, toluene, and isomers of xylene) and hydrocarbon in mineral oil, required addition of nitrate to the water-contaminated sites due to the lack of an electron acceptor in the water (Gersberg *et al.*, 1993; Hunkeler *et al.*, 1995). Fukui et al. (1999) also reported the addition of nitrate to oil contaminated-site containing saturated and aromatic hydrocarbons as a potential mean for enhancing biodegradation effort on-site. Therefore, anaerobic biodegradation of NAs, with addition of electron acceptors into tailing ponds, could be a potential alternative treatment in tailing ponds (especially in anaerobic stabilization ponds).

To date, there is little information available on anaerobic biodegradation of NAs in oil sand tailings ponds waters. Some groups observed a significant production of methane in oil sands tailings ponds, indicating that there is a possibility of anaerobic biodegradation of NAs, even though it is still not clear if NAs was driving the methanogenesis in oil sands tailings ponds (Holowenko *et al.*, 2001; Siddique *et al.*, 2011). Misiti et al. (2013) reported that NAs were not degraded under denitrifying condition, although nitrate removal rate and nitrogen gas production increased as initial concentration of NAs was increased. Additionally it was reported that under denitrification condition with high concentration of NAs bacteria underwent significant cell lysis,

resulting in production of ammonia (Misiti *et al.*, 2013). Musat et al. (2010) studied the anaerobic biodegradation of cyclohexane and ammonia from petroleum and refined petroleum products and reported that denitrification process occurred, accompanied by anaerobic ammonium oxidation (Annamox).

In the past, many studies have been conducted on biodegradation of NAs and various factors including molecular structure of NAs, sediment structure, temperature, pH, dissolved oxygen, nutrients, and bacteria/organisms types have been identified as the factors influencing the biodegradation of NAs (Clemente *et al.*, 2004; Han *et al.*, 2008; Kannel and Gan, 2012). To date, however, there have not been many works on anaerobic biodegradation of NAs. Thus, the present work focused on anaerobic biodegradation of NAs, with trans-4MCHCA as the model compound.

Study of biodegradation of trans-4MCHCA under denitrifying condition in the current work was valuable as it revealed anaerobic biodegradation can be used as a potential treatment for NA contaminated waters which could eliminate aeration cost associated with aerobic biodegradation. Also, it can be used as an in-situ treatment alternative in stabilization pond amended with nitrate. Present work investigated the effect of initial concentration of trans-4MCHCA and temperature in batch system. Effects of trans-4MCHCA loading rate and trans-4MCHCA feed concentration were also examined in continuous systems. Two different continuous reactors: CSTR and biofilm, were utilized and their performances were compared. Finally, to assess the effectiveness of anaerobic treatment, comparison of anaerobic biodegradation with aerobic biodegradation were conducted.

CHAPTER 3
MATERIALS AND METHODS

3.1. Surrogate Naphthenic Acid

Previous works reported that there were three major sources of naphthenic acids used in various studies: commercially available NAs, those extracted from oil sands process-affected waters, and individual surrogate naphthenic acids (Holowenko *et al.*, 2001). In the current study, trans-isomer of 4-methyl-1-cyclohexane carboxylic acid (referred to as trans-4MCHCA, Sigma Aldrich, 98% purity, CAS No. 13064-83-0) was selected as a candidate NA compound because of its commercial availability, molecular structure, and available biodegradation data under aerobic conditions (Paslawski *et al.*, 2009). Trans-4MCHCA has an appearance of white crystal solid at room temperature. The molecular formula of trans-4MCHCA is $CH_3C_6H_{10}COOH$ with molecular weight of 142.2 g mol^{-1}. The molecular conformation and molecular structure of trans-4MCHCA is presented in the Figure 3.1.

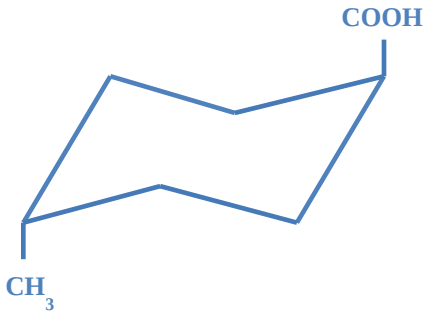


Figure 3.1. Molecular structure of trans-4-methyl-1-cyclohexane carboxylic acid (Sigma-Aldrich)

3.2. Microbial Culture and Medium

The original mixed culture used in this study had been enriched, from previous study, from the soil of a site contaminated with heavy hydrocarbon, using a commercially prepared naphthenic acid (NA) mixture (Fluka technical NAs, Sigma Aldrich, CAS No, 1388-247-5) as substrate and under aerobic condition (Paslawski *et al.*, 2009). The mixed culture was then acclimated to anaerobic biodegradation of NAs using sterilized modified McKinney's medium containing 100 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate (10 mM) as the terminal electron acceptor. Acclimation was carried out in 150 mL serum bottles containing 100 mL of McKinney's medium which was purged with nitrogen gas for 5 minutes to ensure the absence of oxygen. The nitrogen was introduced to the medium through an Acro 50, PFTE 0.2 µm (VWR), followed by a nylon membrane with pore diameter of 0.2 µm (VWR) to maintain the sterile conditions of the medium. Trans-4MCHCA (100 mg L^{-1}) was then added to medium and dissolved by vigorous mixing for three hours prior use. Nitrate was added in the form of potassium nitrate (KNO_3) powder at a concentration of ~ 1 g L^{-1} (10 mM). Following the nitrogen purging, serum bottles were capped with rubber septum and aluminum seal. The microbial culture grown previously under aerobic conditions was used as initial inoculum and added to each bottle (10% v/v). Optical density, concentration of trans-4MCHCA, and concentrations of nitrate and nitrite were then monitored by regular sampling. Cultures were placed on the bench (no mixing to prevent air diffusion) and maintained at room temperature (24 ± 2ºC). Following the complete biodegradation of trans-4MCHCA (approximately fourteen days after inoculation), the developed culture was used as inoculum for subculturing under similar conditions. Subculturing was carried out every 14 days in 150 mL anaerobic serum bottles containing 100 mL of sterilized medium with 100 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} (10 mM) nitrate. After several

subculturing, the developed culture showed reproducible profiles for biodegradation of trans-4MCHCA and reduction of nitrate with complete biodegradation of 100 mg L^{-1} trans-4MCHCA achieved over a much shorter period of five days. A previous work on analysis of the microbial culture conducted at commercial laboratory (EPCOR-Quality Assurance Lab, Edmonton, Canada) identified two dominant species of microorganisms in the culture: *Pseudomonas aeruginosa* and *Variovorax paradoxus* (Huang *et al.*, 2011). These two species have been known for their ability to degrade recalcitrant organic compounds in the environment (Huang, 2011; D'souza, 2012).

McKinney's modified medium (Hill & Robinson, 1975; Paslawski *et al.*, 2009) was used for growth of microbial consortium, culture maintenance, and in biodegradation studies. McKinney's modified medium was composed of various nutrients with the following composition: KH_2PO_4 (840 mg L^{-1}); K_2HPO_4 (750 mg L^{-1}); $(NH_4)_2SO_4$ (474 mg L^{-1}); NaCl (60 mg L^{-1}); $CaCl_2$ (60 mg L^{-1}); $MgSO_4.7H_2O$ (60 mg L^{-1}); $Fe(NH_4)_2(SO_4)_2.6H_2O$ (20 mg L^{-1}). Trace medium was added to the McKinney's modified medium at 0.1% (v/v) to support the growth and activity of the bacteria used during biodegradation process. Trace medium had composition of: H_3BO_3 (600 mg L^{-1}); $CoCl_2$ (400 mg L^{-1}); $ZnSO_4.7H_2O$ (200 mg L^{-1}); $MnCl_2$ (60 mg L^{-1}); $NaMoO_4.2H_2O$ (60 mg L^{-1}); $NiCl_2$ (40 mg L^{-1}); and $CuCl_2$ (20 mg L^{-1}). All medium components were dissolved in reverse osmosis water (RO water). The medium was sterilized prior to use at 121 °C for 15 minutes. The modified McKinney's medium was selected based on the previous studies with naphthenic acids (Paslawski, 2009; Huang, 2011; D'souza, 2012).

3.3. Batch Biodegradation of trans-4MCHCA under Anaerobic Conditions

Following the establishment of the microbial culture under anaerobic conditions, batch experiments were conducted to study the microbial growth, biodegradation, and nitrate reduction kinetics under anaerobic condition. The effects of trans-4MCHCA concentration and temperature were investigated. Batch experiments were carried out in 150 mL serum bottles containing 100 mL of sterilized modified McKinney's medium (described previously) with trans-4MCHCA and nitrate at desired concentrations. The serum bottles were prepared according to procedure described in section 3.2. Four different initial concentrations of trans-4MCHCA in the range of 100 to 750 mg L^{-1} (~100, 250, 500, and 750 mg L^{-1}) were tested. The evaluated concentration range was significantly higher than naphthenic acids concentrations in tailing ponds waters (40 – 120 mg L^{-1}). High concentration of trans-4MCHCA was tested to study the capability of the microbial system, biodegradation kinetics, and kinetics of denitrification processes.

The concentration of nitrate was adjusted according to the concentration of trans-4MCHCA. Nitrate was added in the form of potassium nitrate (KNO_3) and initial concentration of nitrate varied from 620 mg L^{-1} to 1,860 mg L^{-1}, depending on the concentration of trans-4MCHCA used (i.e. with higher initial concentration of trans-4MCHCA, higher concentration of nitrate were needed and used). For instance, complete biodegradation of 100 mg L^{-1} of trans-4MCHCA required approximately 434 mg L^{-1} (7 mM) of nitrate, while for 250, 500 and 750 mg L^{-1} trans-4MCHCA, 620 mg L^{-1} (10 mM), 1,240 mg L^{-1} (20 mM), and 1,860 mg L^{-1} (30 mM) nitrate were needed, respectively. Each bottle was inoculated by a 7-day old culture (10% v/v). Serum bottles were maintained at room temperature (24 ± 2°C) and were placed on the bench (no mixing to prevent air diffusion). Samples were taken on daily basis, started immediately after inoculation.

Bottles were gently shaken prior to sampling. The samples were analyzed for trans-4MCHCA, nitrate and nitrite concentrations, and optical density. Optical density was measured and then converted to biomass concentration. The sampling frequency was increased during exponential phase and sampling was carried out until the stationary growth was achieved (no change in trans-4MCHCA and nitrate concentrations). The progressive experiments were carried out using the preceding batch culture, as an inoculum, in order to permit adaptation of the microbial consortium to higher substrate concentration. Experiments were carried out in duplicates. The average value of the data and associated standard deviations were used to present the results.

Upon completion of concentration experiments at room temperature (24 ± 2°C), additional batch experiments were carried out to assess the effect of temperature on biodegradation using temperature controlled environmental chamber. Using the preceding culture with initial trans-4MCHCA concentration at 250 mg L^{-1} and initial nitrate concentration at 620 mg L^{-1} (10 mM), temperature experiments were carried out in environmental chambers by setting up chamber's temperature to initial temperature of 20°C. Temperature, then, decreased to 15 and 10°C. Similarly, temperature was incrementally increased from room temperature (24 ± 2°C) to 30 and 35°C. All the procedures were similar to those described earlier where samples were collected for trans-4MCHCA, nitrate, nitrite, and optical density analysis. Experiments were carried out in duplicates. The average value of the data and associated standard deviations were used to present the results.

Control experiments were also carried out to confirm that biodegradation process of trans-4MCHCA coupled with denitrification process: 1)- duplicate controls of two different

concentration of trans-4MCHCA and its corresponding nitrate (250 mg L^{-1} of trans-4MCHCA with 620 mg L^{-1} of nitrate and 500 mg L^{-1} of trans-4MCHCA with 1,240 mg L^{-1} of nitrate) with the absent of inoculum and 2)- duplicate controls of 250 mg L^{-1} of trans-4MCHCA and 10% (v/v) inoculum with the absent of nitrate as electron acceptor.

3.4. Anaerobic Biodegradation in Continuous Stirred Tank Reactor

Continuous stirred tank reactor (CSTR), used to study the anaerobic biodegradation of trans-4MCHCA, was a glass vessel with diameter of 5.3 cm and height of 14.6 cm. The reactor had two ports for influent (feed) and effluent, and a sampling port. The working volume of the reactor was 200 mL. The influent and effluent ports of CSTR had a diameter of 0.6 cm. Magnetic stirrer was used to provide good mixing and to maintain cells in suspension. Feed was introduced into the reactor using a variable flow peristaltic pump. A schematic of the experimental system is shown in Figure 3.2. Initially, CSTR was run batchwise. One hundred and eighty mL of modified McKinney's medium containing 250 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} (10 mM) of nitrate was placed in the reactor and purged with nitrogen for 15 minutes. Purging of the medium with filtered nitrogen (N_2) gas was necessary to maintain sterile anaerobic condition in the medium and in the reactor. This step was followed by addition of 20 mL of a 7-day old culture (10% v/v) into the reactor. Once complete biodegradation of naphthenic acid was achieved, the CSTR was switched to continuous mode. Fresh sterile anaerobic medium containing trans-4MCHCA (average concentration: 232.5 ± 22.9 mg L^{-1}) and nitrate (average concentration: 613.7 ± 10.5 mg L^{-1} or 9.9 ± 0.2 mM) were pumped into the CSTR at a flow rate of 0.5 mL h^{-1}, using the peristaltic pump. Effluent left the system through an overflow tube. The flow rate was increased incrementally until the cell washout occurred (i.e. significant decrease in

concentration of biomass and sharp increases in concentrations of naphthenic acid and nitrate). Overall 11 flow rates in the range of 0.5 to 192.0 mL h^{-1} (0.5, 1.0, 2.2, 4.7, 10.1, 19.2, 37.4, 89.0, 118.0, 148.1, and 192.0 mL h^{-1}) were tested. Each flow rate was maintained until the system reached steady state condition. Steady state condition was assumed by a consistency in optical density, residual concentration of trans-4MCHCA and nitrate for three to five residence times. The increase in flow rate continued until the system approached washout conditions. Similarly to those described in earlier section, sampling was done on daily basis and in shorter intervals at high flow rates for measurement of optical density, trans-4MCHCA, nitrate, and nitrite concentrations. The average values of optical density, trans-4MCHCA concentration, and nitrate concentration and associated standard deviations for the samples collected over three residence times following the establishment of steady state condition were calculated and used to present the result. Noted here, CSTR refers to a continuous flow stirred tank reactor.

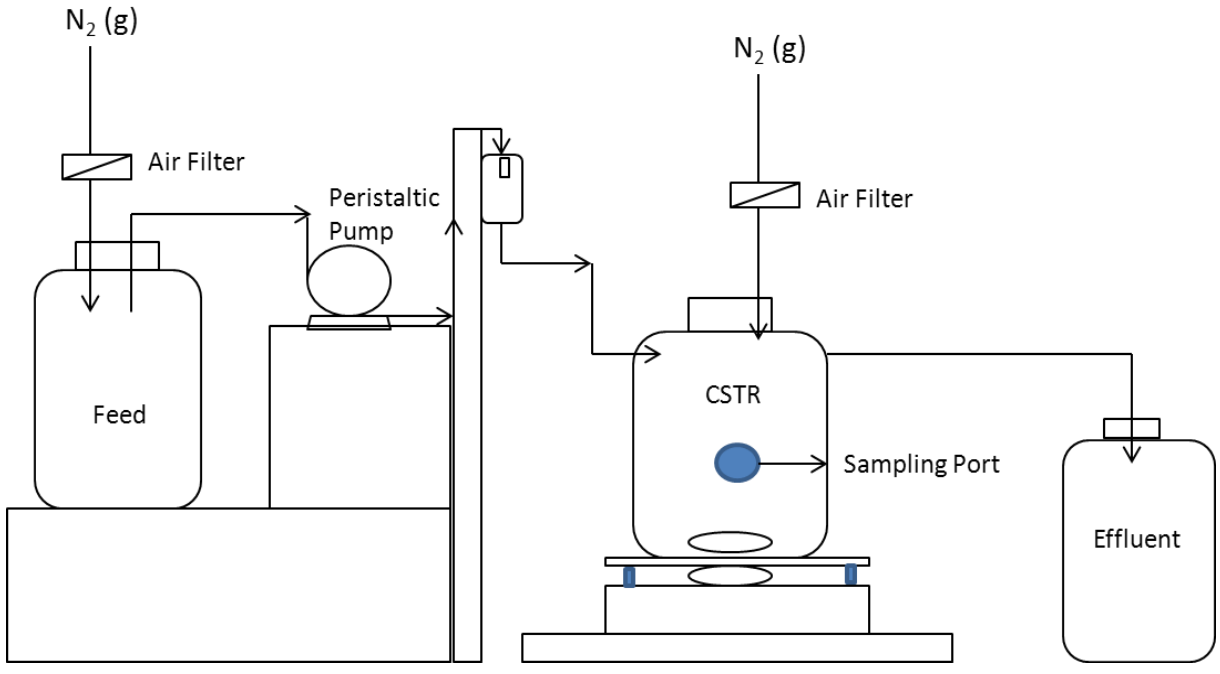
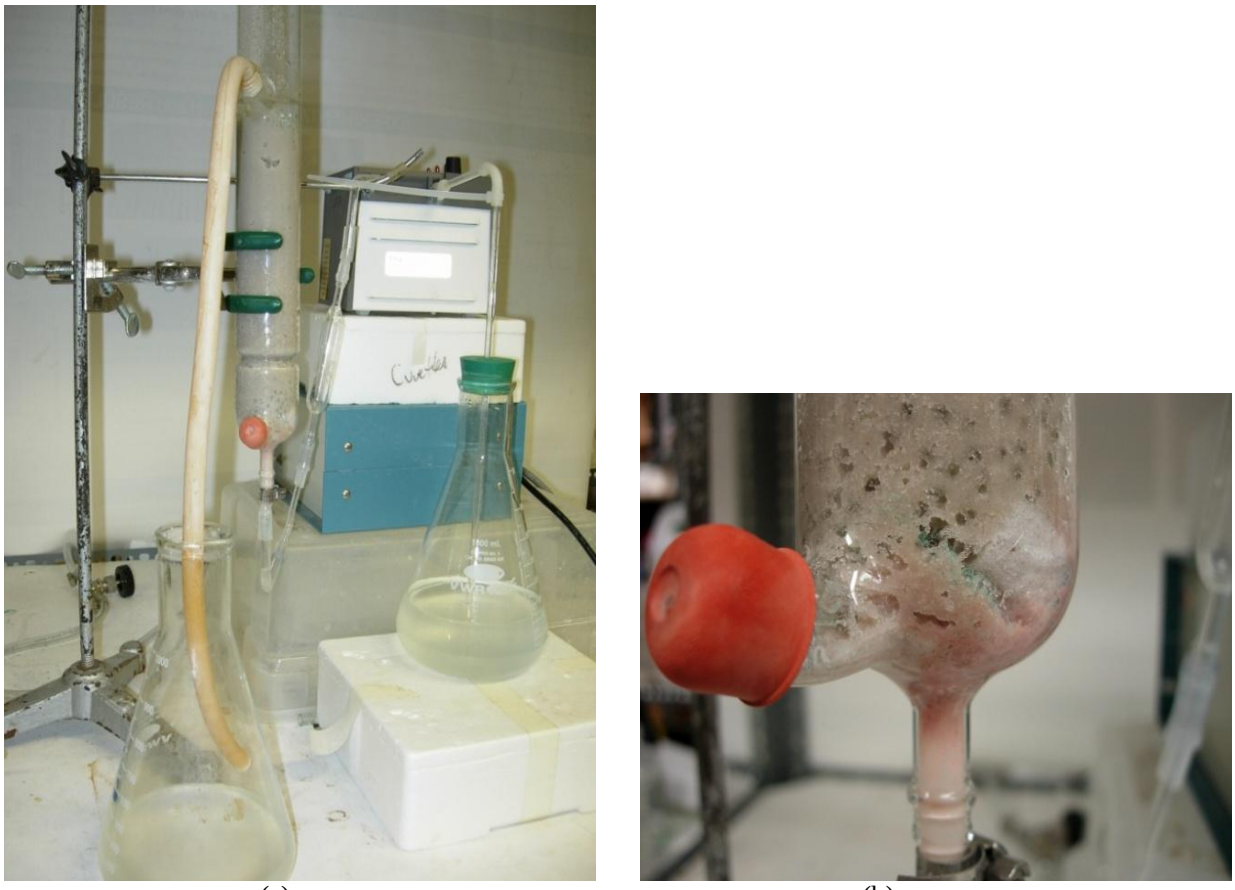


Figure 3.2. Schematic diagram of experimental set-up of continuous stirred tank reactor (CSTR)

3.5. Anaerobic Biodegradation in the Biofilm Reactor

The continuous biodegradation of trans-4MCHCA was also studied at the room temperature under anaerobic condition in a biofilm reactor. The biofilm set-up consisted of a glass column served as the biofilm reactor, feed pump, feed vessel, and effluent vessel. Sand with average diameter of 225 μm (mesh size: -50 to +70) was used as carrier for establishment of the biofilm. Glass beads and sponge were placed at the bottom of the column to support the sand particles. The beads had diameter approximately about 1.0 mm. The effluent port had diameter of 1.2 cm, while the influent port had diameter of 1.0 cm. The bioreactor had height of 28.0 cm, diameter of 4.4 cm, and a working volume of 25 mL. The working volumes were determined at the beginning and at the end of experiments as 26 and 24 mL respectively. The average value of 25 mL was used in the calculation of residence time, loading, and removal rates of trans-4MCHCA and nitrate. Biofilm was developed through a period of 4 weeks by trickling sterile modified McKinney's medium containing 100 mg L^{-1} of octanoic acid, 620 mg L^{-1} (10 mM) of nitrate, and 10% (v/v) of inoculum at flow rate of 0.5 mL h^{-1}. Octanoic acid was used as the initial substrate because it has a linear structure and could be biodegraded easily. This assisted in rapid development of biofilm in the reactor. The feed was purged with nitrogen to maintain anaerobic conditions in the feed and bioreactor. Following the period of 4 weeks, octanoic acid was substituted by trans-4MCHCA with average concentration of 50.0 ± 3.3 mg L^{-1} and nitrate concentration was decreased to an average value of 316.0 ± 22.8 mg L^{-1} (5.1 ± 0.4 mM). A concentration of 50 mg L^{-1} for trans-4MCHCA was consistent with what had been used in an earlier work for aerobic biodegradation and made it possible to make a direct comparison of these two modes of biodegradation (Huang, 2011; D'souza, 2012). It should be pointed out that higher concentrations of naphthenic acids were also tested in the biofilm reactor toward the end

of experimental runs as described in the following section. The biofilm reactor was fed initially at a low flow rate of 0.5 mL h^{-1} to allow acclimation of the biofilm to biodegradation of trans-4MCHCA. Residual concentrations of trans-4MCHCA, nitrate, and nitrite were monitored until residual concentration of trans-4MCHCA dropped to zero. Following this, the biofilm reactor was operated at the same flow rate for several additional days to ensure establishment of steady state conditions. Flow rate of the feed was then increased incrementally. Overall 14 different flow rates ranging from 0.5 to 238.2 mL h^{-1} (0.5, 3.1, 4.6, 9.6, 17.7, 38.8, 60.2, 82.9, 110.2, 146.3, 163.9, 184.1, 217.9, and 238.2 mL h^{-1}) were tested in the biofilm reactor with each flow rate was maintained until the system reached steady state condition, indicated by stable values for residual concentration of trans-4MCHCA and nitrate for three to five residence times (or at least two-three days). Figure 3.3 shows the biofilm reactor set-up.



(a) (b)

Figure 3.3. Photograph of the biofilm reactor and its accessories (a); biofilm can be clearly seen at the bottom of the reactor (b).

After completion of experiments with variable flow rate, biofilm system was used to study effect of initial concentration of trans-4MCHCA at a constant feed flow rate. The various initial concentrations of trans-4MCHCA tested were 93.8 ± 3.6, 237.7 ± 16.8, and 592.7 ± 9.9 mg L^{-1}. An increase in trans-4MCHCA concentration required higher amount of nitrate, thus nitrate concentrations utilized in this part of study were adjusted to provide sufficient electron acceptor for reduction-oxidation (redox) reactions. The concentrations of nitrate used for 93.8, 237.7 and 592.7 mg L^{-1} of trans-4MCHCA were 283.5 ± 8.1 mg L^{-1} (4.6 ± 0.1 mM), 619.4 ± 29.8 mg L^{-1} (10.0 ± 0.5 mM), and 912.7 ± 93.0 mg L^{-1} (14.7 ± 1.5 mM), respectively. For the concentration of 93.8 mg L^{-1} of trans-4MCHCA and 283 mg L^{-1} of nitrate corresponding feed flow rate was

116.7 mL h^{-1}, while at concentration of 237.7 mg L^{-1} trans-4MCHCA with 619.4 mg L^{-1} nitrate and 592.7 mg L^{-1} trans-4MCHCA with 912.7 mg L^{-1} nitrate corresponding feed flow rates were 110.3 and 110.1 mL h^{-1}, respectively (i.e. it was not possible to run the system at exactly identical flow rates). Sampling was carried regularly in similar manner to that of CSTR. Again average data and associated standard deviations in samples taken following the establishment of steady state conditions were calculated and used to present the results.

3.6. Analytical Methods

3.6.1. Naphthenic Acids Concentration

Several techniques have been used to measure the concentration of naphthenic acids, including: high-performance liquid chromatography (HPLC), high-resolution mass spectrophotometer (HPLC/HRMS), gas chromatography-mass spectrophotometry (GC/MS), fourier transform infra-red (FT-IR) spectroscopy, high-performance liquid chromatograph quantitative quadrupole time of flight-MS (HPLC/QTOF-MS), liquid secondary ion mass spectrometry (LSI-MS), atmospheric pressure ionization (API)-MS, and electrospray ionization (ESI) (Jones *et al.*, 2001; Holowenko *et al.*; 2002, Bataineh *et al.*; 2006, Paslawski, 2008; Han *et al.*, 2009; Grewer *et al.*, 2010). Based on the earlier works, gas chromatography with flame ionization detector (GC-FID) and a split/splitless injector was identified as a suitable approach to analyze the trans-4MCHCA concentration in aqueous solution and used in the present work (Paslawski *et al.*, 2008; Huang *et al.*, 2011; D'souza, 2012). The gas chromatograph was a Varian-430 with a HP-INNOWAX high resolution gas chromatography column (19091N-133). The GC column had the following specification: length of 30 m, inside diameter of 0.25 mm, and film thickness of 0.25 µm. Helium gas (He) was used as carrier. The system was operated under following conditions: H_2

flow rate of 30 mL min^{-1}; He flow rate of 29 mL min^{-1}; air flow rate of 300 mL min^{-1}; injector temperature of 220°C, and detector temperature of 250°C. The injector was operated in splitless mode with initial temperature of column of 90°C which then increased to 210°C at rate of 40°C min^{-1}. The run time of the system, for each injection, was 5.5 minutes. The retention time for trans-4MCHCA was 3.6 ± 0.2 minutes.

Samples required for analyses were collected using a stainless steel needle and glass hypodermic syringe. Samples were then filtered through a 0.22 µm pore diameter GE magna nylon membrane filter, with diameter of 25 mm, using a stainless steel cartridge. Samples were transferred into 2 mL vials and analyzed immediately to determine trans-4MCHCA concentration. Samples were analyzed in triplicate (three injections to GC), where each sample had injection volume of 0.5 µL. Three injections of Millipore water was carried out after every five samples (15 injections) to prevent the potential accumulation of the NA in the column. At the end of analysis, samples were stored immediately at -80°C for potential future reanalysis. A linear calibration curve was generated using six standard solutions of trans-4MCHCA (10, 25, 50, 70, 100 and 125 mg L^{-1}) in the sterile modified McKinney's medium. The correlation coefficient of calibration curve was 99.62%.

3.6.2. Nitrate and Nitrite Concentrations

Nitrate and nitrite concentrations were measured simultaneously using a Dionex ICS-2500 ion chromatograph (IC) with a conductivity detector (CD25A) equipped with an IonPac GC5A guard column and an IonPac CS5A analytical column. ICS-2500 has eluent of 1.0 mM KOH with eluent flow rate that was set at 1.0 mL h^{-1}. The IC system was calibrated using standard solutions

of both nitrite and nitrate with concentrations ranging from 5 to 50 mg L^{-1} (5, 10, 20, 30, and 50 mg L^{-1}). Establishment of quadratic calibration curves for each ion was done by three injections of each concentration (with injection volume of 25.0 µL). The correlation coefficients for nitrate and nitrite calibration curves were 99.97% and 99.68%, respectively.

Samples were taken in the same way as those for GC analysis through filtration process with GE magna nylon membrane filter. After filtration, 0.1 mL of samples was diluted with 0.9 mL of Milipore water (10 times dilution). Samples then were further diluted to the desired concentration ranges for IC calibration (5 – 50 mg L^{-1} of nitrate and nitrite). At the end of analysis, the 10 times diluted samples were preserved in -80°C freezer for reanalysis (if necessary).

3.6.3. Biomass Measurement

A Mini Shimadzu (model 1240) ultraviolet (UV) spectrophotometer was utilized to determine the optical density (OD) as a representative of biomass concentration. Wavelength of 620 nm was applied to measure the OD of the solution and modified McKinney's medium was utilized as blank. A calibration curve, generated previously used to determine the biomass concentration. The correlation coefficients of biomass calibration curves were 99.91%.

3.6.4. Reproducibility and Data Uncertainty

Some of the batch experiments were carried out in duplicates to determine the reproducibility of the results. For these experiments the results have been presented using the average values of the data and associated standard deviations (shown as error bars) obtained in the duplicate

experiments. In case of continuous experiments (both CSTR and biofilm) following the establishment of steady state at each applied flow rate the experiments were continued for three to five additional residence times at the same flow rate and samples were collected. The average value of the data and associated standard deviation obtained from these samples were used to present the results.

CHAPTER 4
RESULTS AND DISCUSSION

This chapter presents the results of anaerobic biodegradation of trans-4MCHCA in both batch and continuous systems. The effect of initial concentration of trans-4MCHCA and temperature on biodegradation and denitrification processes, studied in batch system, are presented first. The results that deal with the effects of initial concentration and volumetric loading rate of trans-4MCHCA on biodegradation and denitrification processes in continuously operated stirred tank reactor (CSTR) and biofilm reactor are presented next. Finally, biodegradation of trans-4MCHCA under aerobic and anaerobic condition are compared to verify the effectiveness of these two modes of biodegradation.

4.1. Batch Biodegradation of trans-4MCHCA

Batch experiment of anaerobic biodegradation of trans-4MCHCA was carried out to investigate the effects of two variables: 1)-initial concentration of trans-4MCHCA and 2)- temperature. Data presented in some figures are the average values of data obtained from duplicate experiments and the error bars represent the associated standard deviations.

4.1.1. Effect of trans-4MCHCA Initial Concentration

Biodegradation of trans-4MCHCA and nitrate reduction were studied using various initial concentrations of trans-4MCHCA with range started from 100 to 750 mg L^{-1} (100, 250, 500, and 750 mg L^{-1}). Figure 4.1 and 4.2 demonstrates the profile of biodegradation of NA and denitrification at different initial concentrations of NA.

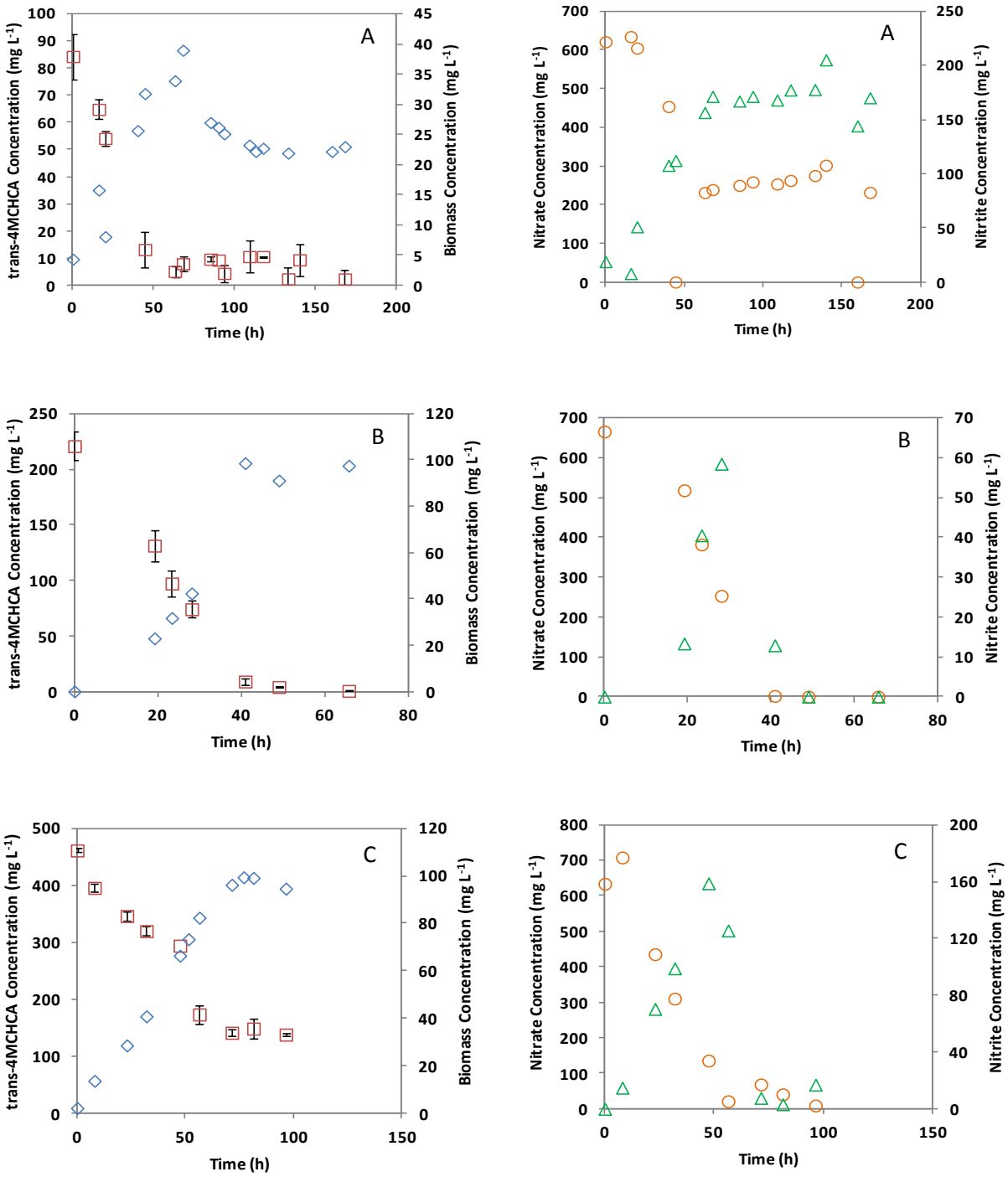


Figure 4.1. Biomass growth,substrates biodegradation, and denitrification profiles obtained with various initial substrate concentration at room temperature (24 ± 2°C). (A) 100 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate, (B) 250 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate, (C) 500 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate. (□ trans-4MCHCA concentration; ◊ biomass concentration; ○ nitrate concentration; Δ nitrite concentration). Error bars represent the standard deviations in the duplicate experiments and might not be visible in some cases.

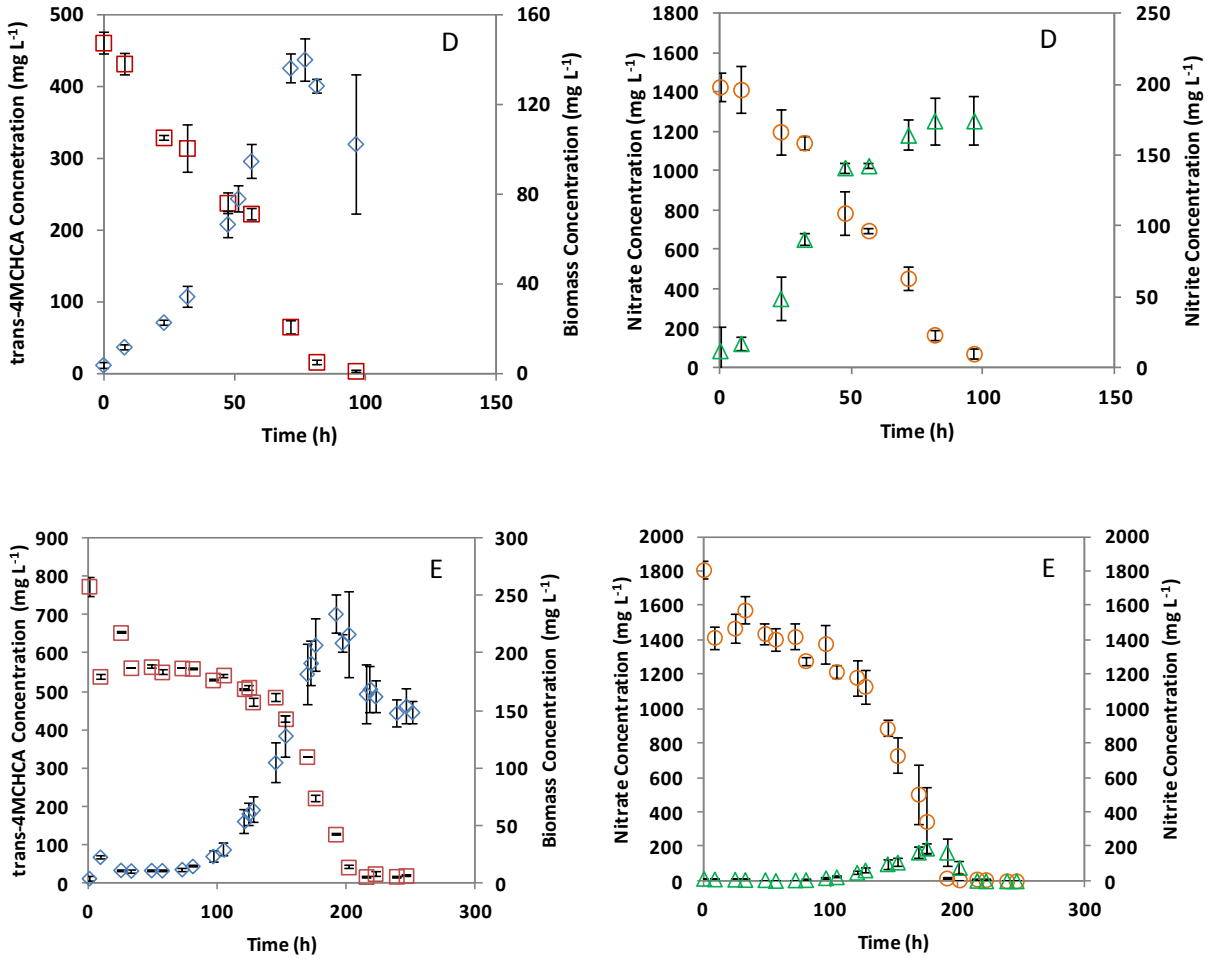


Figure 4.2. Biomass growth, substrates biodegradation, and denitrification profiles obtained with various initial substrate concentration at room temperature (24 ± 2°C). (D) 500 mg L^{-1} of trans-4MCHCA and 1,240 mg L^{-1} of nitrate, (E) 750 mg L^{-1} of trans-4MCHCA and 1,860 mg L^{-1} of nitrate. (□ trans-4MCHCA concentration; ◊ biomass concentration; ○ nitrate concentration; Δ nitrite concentration). Error bars represent the standard deviations in the duplicate experiments and might not be visible in some cases.

A wide range of initial NA concentrations was examined to verify the ultimate potential of microbial culture in biodegradation of trans-4MCHCA. As previously mentioned, nitrate was added to the medium as an electron acceptor. The initial concentration of nitrate in the system was adjusted based on the initial concentration of trans-4MCHCA (varied from 620 mg L^{-1} to 1,860 mg L^{-1}). In all cases, there was a direct relationship between the microbial growth, NA

biodegradation (substrate utilization), and nitrate reduction during the biodegradation process. The biodegradation profiles showed that once cells started to grow (increase in optical density/biomass concentration), the trans-4MCHCA concentration declined, indicated that microorganisms utilized trans-4MCHCA as their carbon and energy source. Figure 4.1 shows that at trans-4MCHCA initial concentration of 100 mg L^{-1}, maximum biomass concentration was approximately 39 mg L^{-1}, while with 750 mg L^{-1} trans-4MCHCA initial concentration (highest trans-4MCHCA concentration tested in this work), the maximum biomass concentration was 234 mg L^{-1} (see Figure 4.2).

Decrease in trans-4MCHCA concentration was accompanied by decrease in nitrate concentration and production of nitrite, an indication of coupling of biodegradation and denitrification processes. The process initiated and proceeded when bacterial enzymatic system caused oxidation of trans-4MCHA and released of electrons. The released electrons were then consumed in denitrification process, resulting in reduction of nitrate ion. Denitrification process involves several steps with the main ones being: 1)- reduction of nitrate to nitrite, where nitrite could be further reduced to nitrogen gas or 2)- reduction of nitrate to nitrogen gas directly (An *et al.*, 2011). Nitrate and nitrite both act as electron acceptors and as a substitute for oxygen which serves as the electron acceptor during the aerobic biodegradation process. Due to coupling of biodegradation and denitrification processes, higher initial concentration of trans-4MCHCA would require higher concentration of nitrate so that complete redox reactions can occur.

As it is shown in Figure 4.1, initial concentration of 100 mg L^{-1} trans-4MCHCA required less than 620 mg L^{-1} of nitrate, while at higher concentration of 250 and 500 mg L^{-1} oxidation of

trans-4MCHCA required 620 and 1,240 mg L^{-1} of nitrate, respectively. Highest concentration tested in this study (750 mg L^{-1}) required the highest amount of nitrate of 1,860 mg L^{-1}. Data presented in Figure 4.1 (C) shows that with 500 mg L^{-1} trans-4MCHCA, the added nitrate at concentration of 620 mg L^{-1} of nitrate was not sufficient for complete biodegradation of trans-4MCHCA and biodegradation stopped as soon as nitrate and produced nitrite were completely consumed. This was evident from the residual concentration of trans-4MCHCA in the system which remained constant at an approximate level of 145 mg L^{-1}. However, at the same initial concentration of substrate (500 mg L^{-1} of trans-4MCHCA), the biodegradation was complete when 1,240 mg L^{-1} nitrate was used instead of 620 mg L^{-1}. Based on these results and in order to prevent the incomplete biodegradation, in the batch culture with 750 mg L^{-1} trans-4MCHCA, 1,860 mg L^{-1} nitrate was provided which alleviate the observed problem (Figure 4.2, panel D).

Degradation of nitrite was detected in some cases (especially when nitrate was completely utilized), showing utilization of nitrite by the microbial cell and an indication that nitrite also served as an electron acceptor. Duplicate control experiments, with 100 and 500 mg L^{-1} trans-4MCHCA and the required corresponding nitrate concentration, with no inoculum which were carried out for five days showed no decrease in trans-4MCHCA concentration, indicating that abiotic oxidation of trans-4MCHCA did not occur (i.e. chemical oxidation of trans-4MCHCA in the presence of nitrate). Regular measurement of pH indicated an increase in pH from initial value of 6.5 to a final value of 7.5. The slight increase in pH might be the result of consumption of naphthenic acid and denitrification process. This finding strengthen the validity of conclusion that biodegradation of trans-4MCHCA was coupled to denitrification process.

As shown in both Figure 4.1 and 4.2, increase in initial concentration of trans-4MCHCA resulted in longer lag phase in the system (an indication of adaptation period of microorganisms). For example, in batch experiment with 100 mg L^{-1} trans-4MCHCA, no lag phase was observed, while in system with 750 mg L^{-1} of trans-4MCHCA, lag phase of 104 h was observed. Increase of lag phase in the higher concentration of trans-4MCHCA was reflected to the utilization of nitrate as well. When the trans-4MCHCA was not utilized yet, nitrate was not reduced, thus lag phase in nitrate profile was also observed. The lag phase indicated that cells were trying to adapt to new environment.

Increase in initial concentration of trans-4MCHCA resulted in longer periods for complete biodegradation of substrate. For instance biodegradation of 100 mg L^{-1} trans-4MCHCA required about 63 hours, while with initial concentrations of 500 mg L^{-1} and 750 mg L^{-1}, 97 and 251 hours were required, respectively. The correlation of biomass, trans-4MCHCA, and nitrate concentration profiles and production of nitrite confirmed that biological activity was responsible for removal of trans-4MCHCA and nitrate, and that trans-4MCHCA biodegradation was coupled to nitrate reduction. Using the slope of the linear part of trans-4-MCHCA and nitrate concentration profiles, biodegradation rate of trans-4MCHCA and corresponding nitrate reduction rate were calculated at different NA initial concentrations and presented in Table 4.1. As indicated, these rates were calculated using the concentration profiles during the exponential phase of microbial activity as it is customary for biological systems (lag phase and stationary phase were excluded). Included in this table is also production rate of nitrite which was calculated using the nitrite concentration profiles.

As seen in Table 4.1, the calculated trans-4MCHCA biodegradation rate ranged from 1.4 mg L^{-1} h^{-1} at the lowest concentration of 100 mg L^{-1} to 5.3 mg L^{-1} h^{-1} at the highest concentration of 750 mg L^{-1}, with the corresponding nitrate reduction rates being 8.5 and 16.4 mg L^{-1} h^{-1}, respectively. These data showed that application of higher initial concentrations of trans-4MCHCA increased the biodegradation rate of trans-4MCHCA and reduction rate of nitrate. This pattern also indicated that trans-4MCHCA at the highest concentration tested in this work did not impose an inhibition effect on the microbial activity. It should be pointed out that biodegradation rate increased sharply when initial concentration of trans-4MCHCA was increased from 100 mg L^{-1} to 250 mg L^{-1}. However, biodegradation rate at initial concentrations of 250, 500 and 750 mg L^{-1} were close. Data in Table 4.1 also shows that for trans-4MCHCA concentration of 250 mg L^{-1}, nitrite was produced at the lowest rate (1.9 mg L^{-1} h^{-1}) indicating that in this case, bacteria might have utilized nitrite simultaneously with nitrate (as secondary electron acceptor), due to insufficient amount of nitrate available in the system.

Table 4.1. Effect of initial concentration of trans-4MCHCA on its biodegradation rate, reduction rate of nitrate, and production rate of nitrite[1]

Trans-4MCHCA Concentration (mg L^{-1})	Nitrate Concentration (mg L^{-1})	Biodegradation Rate of trans-4MCHCA (mg L^{-1} h^{-1}) (R^2 value)	Reduction Rate of Nitrate (mg L^{-1} h^{-1})	Production Rate of Nitrite (mg L^{-1} h^{-1})
100	620	1.4 (0.96)	8.5 (0.89)*	2.5
250	620	4.7 (0.97)	16.4 (0.92)	1.9
500	1240	5.1 ± 0.3 (0.97)	15.3 ± 0.4 (0.98)	2.5
750	1860	5.3 ± 0.1 (0.93)	16.3 ± 0.1 (0.99)	2.3

*The number in the brackets represent R^2 values.

[1] Standard deviation is only given for duplicate experiment

4.1.2. Effect of Temperature

The effect of temperature on biodegradation of trans-4MCHCA under denitrifying condition was evaluated in the present work. The results for biodegradation of 250 mg L^{-1} trans-4MCHCA in the presence of 620 mg L^{-1} nitrate at six different temperatures of 10, 15, 20, 24, 30, and 35°C are presented in Figures 4.3 and 4.4. Similar to the results where effect of initial concentration was investigated, an increase in biomass concentration was accompanied by decrease in concentrations of trans-4MCHCA and nitrate and production of nitrite. Temperature is considered to be one of the important parameter in biodegradation. The results showed that change of temperature resulted in different lag phase. For example, a lag phase of approximately 73 and 24 hours was observed at temperature of 10° and 15°C, while at temperature of 20° and 24 ± 2°C, lag phase was not detected. When temperature was further increased to 30° and 35°C, lag phase of 20 hours were observed again.

Temperature also affected the time required for complete biodegradation of NA and reduction of nitrate (i.e. longer period of time was required at low temperatures of 10 and 15°C). Figure 4.3 shows that at 10° and 15°C, microbial consortium took approximately 950 and 400 hours to complete the biodegradation of trans-4MCHCA. On the other hand, it required only 52 and 49 hours to biodegrade the substrate at temperature of 20° and 24 ± 2°C. Again, as the temperature was increased from room temperature (24 ± 2°C) to 30° and 35°C, increase in the required biodegradation time was observed. These results indicated a temperature in the range 20 - 24°C as the optimum temperature for microbial activity and biodegradation of naphthenic acid in question. Concentration of biomass and its maximum level was also affected by temperature. At the optimum temperature of 20 to 24°C, the maximum biomass concentration was 98.6 mg L^{-1},

while at low temperatures of 10º and 15ºC, the maximum biomass concentration achieved was approximately in the range of 30 to 40 mg L^{-1}. Maximum biomass concentrations at temperatures of 30º to 35ºC was approximately 50.0 to 55.0 mg L^{-1} and again were lower than the level obtained at optimum temperature range.

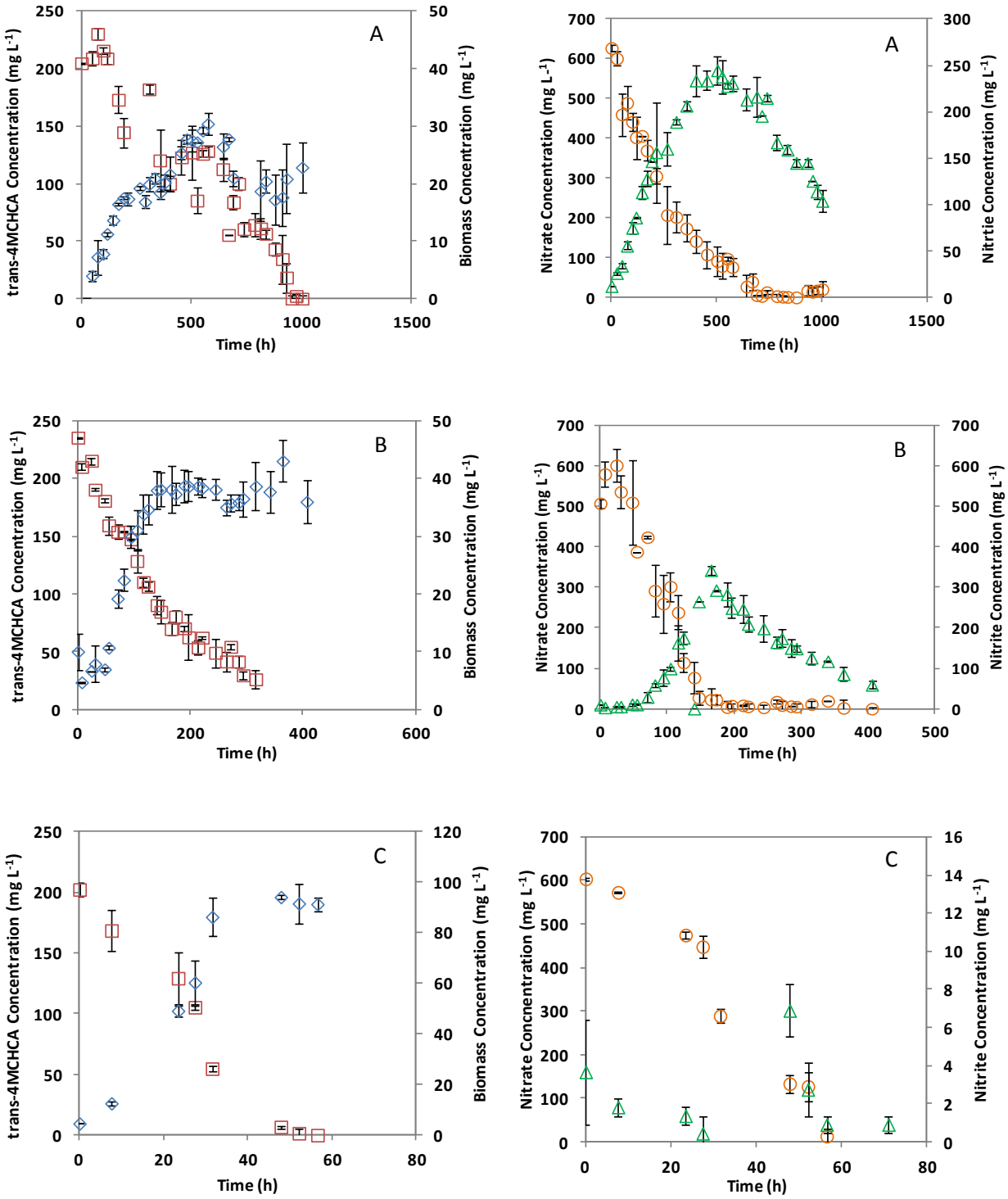


Figure 4.3. Biomass growth,substrates biodegradation and denitrification profiles obtained at various temperatures with 250 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate. (A) 10°C, (B) 15°C, (C) 20°C. (□ trans-4MCHCA concentration; ◊ biomass concentration; ○ nitrate concentration; Δ nitrite concentration). Error bar represents the standard deviations in duplicate experiments which might not be visible in some cases.

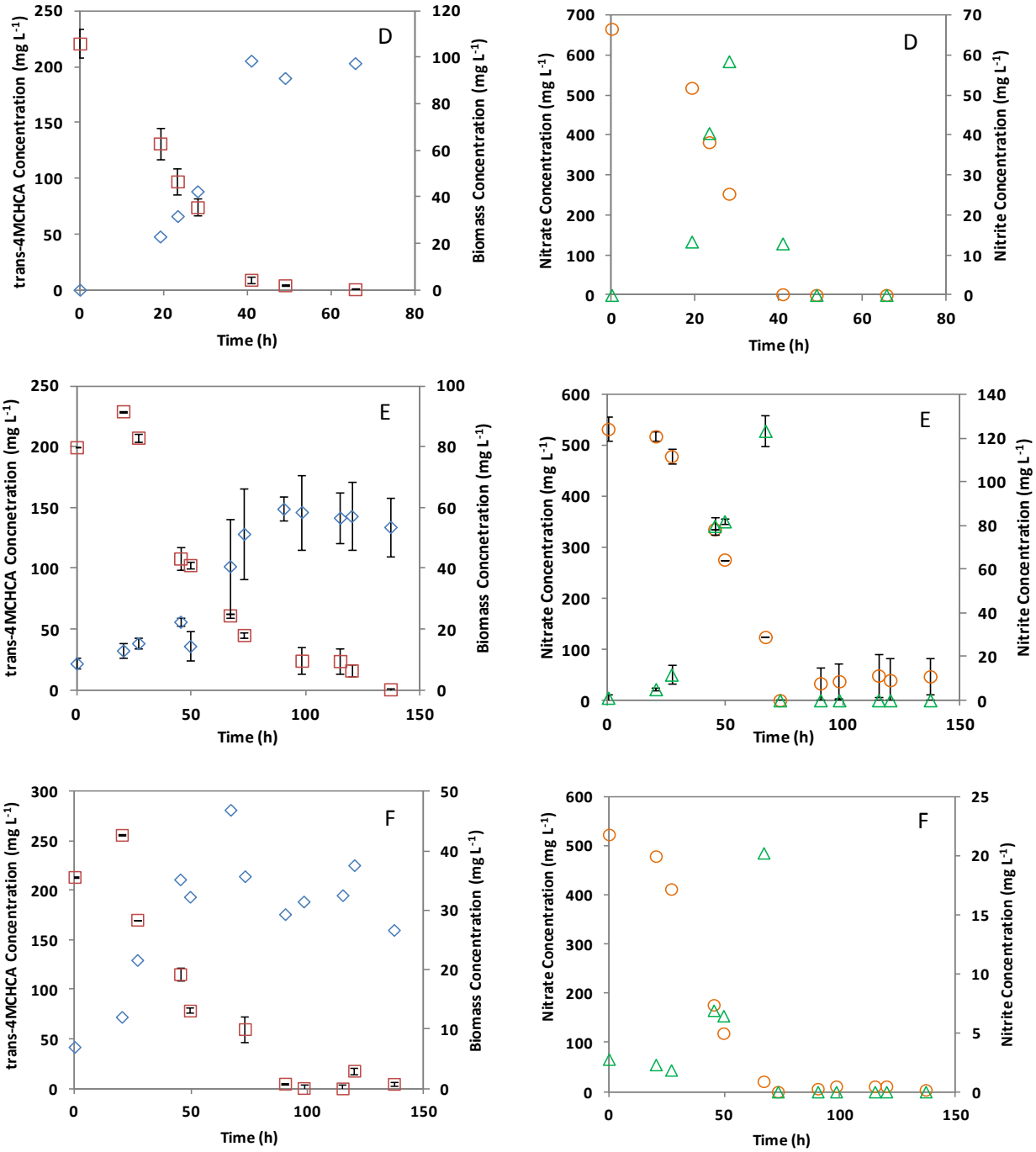


Figure 4.4. Biomass growth, substrates biodegradation, and denitrification profiles obtained at various temperature with 250 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate. (D) 24 ± 2°C, (E) 30°C, (F) 35°C. (□ trans-4MCHCA concentration; ◊ biomass concentration; ○ nitrate concentration; Δ nitrite concentration). Error bar represents the standard deviations in duplicate experiments and might not be visible in some cases.

The profile of nitrate reduction in batch system under various temperatures was found to be similar to the biodegradation profile of trans-4MCHCA. It took longer for the bacteria to reduce nitrate when the system was not run at optimum temperature (20 – 24ºC). As it is seen in Figure 4.3, there was a significant production of nitrite at low temperature (10º and 15ºC), potentially due to slower biodegradation rate. However, at higher temperatures (Figure 4.4), there was little production of nitrite, which indicated that microorganisms utilized nitrite at the same time as nitrate when they were maintained at the optimum temperature. In all cases, it was noticed that once concentration of nitrate dropped to zero, microorganisms started to use nitrite as secondary electron acceptor to further oxidize trans-4MCHCA, thus there was a decrease in nitrite concentration.

Table 4.2. Temperature effect on the biodegradation rate of trans-4MCHCA, reduction rate of nitrate, and production rate of nitrite[2]

Temperature (°C)	Biodegradation rate of trans-4MCHCA (mg $L^{-1} h^{-1}$) (R^2 value)	Reduction rate of nitrate (mg $L^{-1} h^{-1}$) (R^2 value)	Production rate of nitrite (mg $L^{-1} h^{-1}$)
10	0.2 ± 0.1 (0.91)	1.1 ± 0.1(0.94)	0.6
15	0.6 ± 0.1(0.93)	4.2 ± 0.4(0.94)	3.1
20	4.1 ± 0.3 (0.96)	11.0 ± 0.4 (0.94)	0.3
24 ± 2	4.7 (0.97)	16.4 (0.92)	1.9
30	2.2 ± 0.2 (0.90)	7.3 ± 1.5 (0.98)	2.7
35	2.9 (0.89)	8.5 (0.94)	0.7

Table 4.2 summarizes the biodegradation rate for trans-4MCHCA, corresponding nitrate reduction rates, and nitrite production rates observed at various temperatures. The highest rate was observed in batch system at room temperature (24 ± 2ºC), with maximum biodegradation rate of trans-4MCHCA being 4.7 mg L^{-1} h^{-1} and maximum reduction rate of nitrate being 16.4

[2] Standard deviation is only given for duplicate experiments

mg L^{-1} h^{-1}. Table 4.2 confirmed that sub-optimum environmental condition, such as low temperature of 10º and 15ºC and high temperature of 30º and 35ºC, caused a decreased in biodegradation rate of trans-4MCHCA and reduction rate of nitrate. Biodegradation rate of trans-4MCHCA influenced the reduction rate of nitrate, which indicated that biodegradation process of trans-4MCHCA was coupled to reduction rate of nitrate. The low production rate of nitrite at some temperatures suggested that nitrite could be used simultaneously with nitrate as co-electron acceptor.

Duplicate control of experiments with 250 mg L^{-1} trans-4MCHCA, no nitrate, and addition of 10% inoculum were carried out for five days. These batch control experiments showed little change in optical density (biomass concentration) and trans-4MCHCA concentration, confirmed that under anaerobic biodegradation, nitrate was required as an electron acceptor for oxidation of trans-4MCHCA.

4.2. Continuous Biodegradation of trans-4MCHCA in Stirred Tank Reactor

The effect of volumetric loading rate of trans-4MCHCA on anaerobic biodegradation was examined in the CSTR. The performance of the CSTR was assessed in terms of biodegradation rate of trans-4MCHCA and reduction rate of nitrate. The reactor was initially operated under batch condition until the trans-4MCHCA concentration in the reactor decreased to zero. The reactor was then fed with McKinney's medium containing 232.5 ± 22.9 mg L^{-1} trans-4MCHCA and 613.4 ± 10.5 mg L^{-1} nitrate at a flow rate of 0.5 mL h^{-1} (corresponding trans-4MCHCA loading rate: 0.5 mg L^{-1} h^{-1} or residence time: 416.7 h). When 100% removal percentage was achieved, the flow rate was increased stepwise. CSTR showed a general profile in which increase

in dilution rate (increase in loading rate) caused a decrease in biomass concentration and led to an increase in residual concentrations of trans-4MCHCA and nitrate. This is due to the fact that in a continuous system (non-attached biomass), the biomass concentration in the reactor and consequently extent of biodegradation and denitrification processes are controlled by the production rate of biomass as a result of growth and removal of biomass by the influent stream.

An increase in dilution rate led to decrease of biomass and consequently decrease in utilization of trans-4MCHCA and nitrate and production of nitrite. Figure 4.5 shows that initially biomass concentration increased at low dilution rate and reached the maximum value of 178.3 ± 13.6 mg L^{-1} at a dilution rate of 0.051 h^{-1}. This happened due to the low flow rate that allowed microorganisms to consume trans-4MCHCA effectively and grow at a rate faster than their removal by the effluent. When the dilution rate was further increased, the cells in CSTR started to get washed out, thus biomass concentration decreased and finally dropped to a very low concentration (3.0 mg L^{-1}). Figure 4.6 shows the nitrate and nitrite concentration profiles in CSTR. At low dilution rate, concentration of nitrate stayed low due to the consumption of electron acceptor by the microorganisms; this caused the concentration of nitrite in CSTR to increase and reach its maximum value of 22.5 ± 0.9 mg L^{-1}.

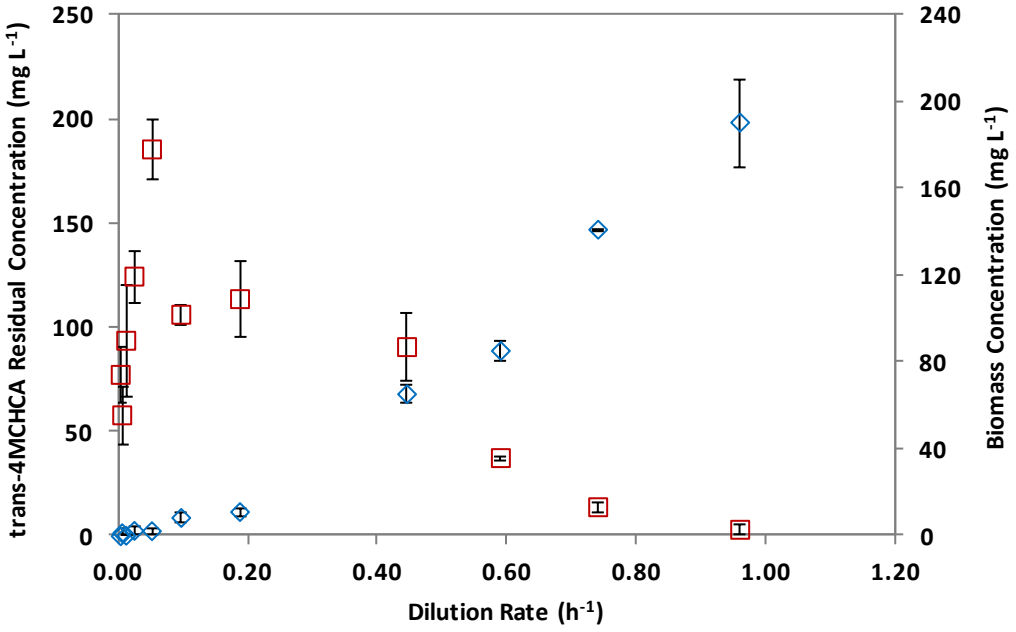


Figure 4.5. Steady-state profiles of trans-4MCHCA and biomass concentrations observed in continuous stirred tank reactor (CSTR). (◊ trans-4MCHCA residual concentration; □ biomass concentration). Values reported are the average data for multiple samplings of the reactor over an extended period equal to three to five residence times after the establishment of steady state. Error bars represent the standard deviations and might not be visible in some cases.

As the dilution rate was increased, the concentration of nitrate in CSTR started to increase (due to lower number of cells available to utilize NA and consequently nitrate as the electron acceptor), thus production of nitrite decreased and concentration of nitrite in CSTR dropped to zero (Figure 4.6).

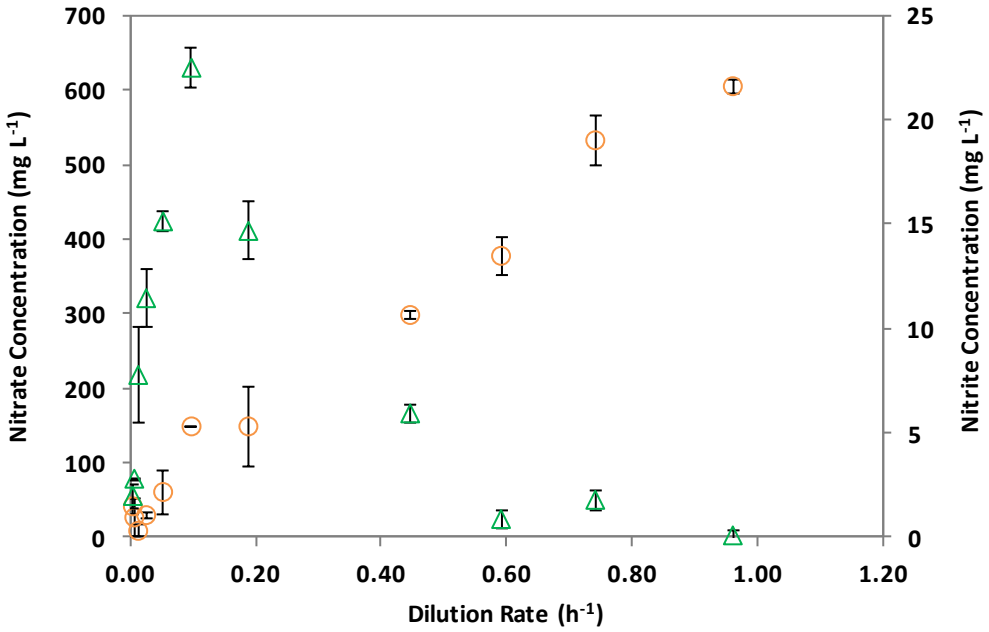


Figure 4.6. Steady-state profiles of nitrate and nitrite concentrations observed in continuous stirred tank reactor (CSTR). (○ nitrate concentration; Δ nitrite concentration). Values reported are average data for multiple samplings of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state. Error bars represent the standard deviations, which might not be visible in some cases.

Figure 4.7 shows the variation of trans-4MCHCA removal rate and its removal percentage as a function of trans-4MCHCA loading rate in the CSTR. Figure 4.8 shows the variation of nitrate removal rate and its removal percentage as a function of trans-4MCHCA loading rate. As seen, removal rate of both trans-4MCHCA and nitrate increased linearly as trans-4MCHCA loading rate was increased. Maximum removal rate of trans-4MCHCA (105.4 mg L^{-1} h^{-1}) was attained at a trans-4MCHCA loading rate of 157.8 mg L^{-1} h^{-1} (corresponding dilution rate: 0.590 h^{-1}) with corresponding maximum removal rate of nitrate being 144.5 mg L^{-1}.

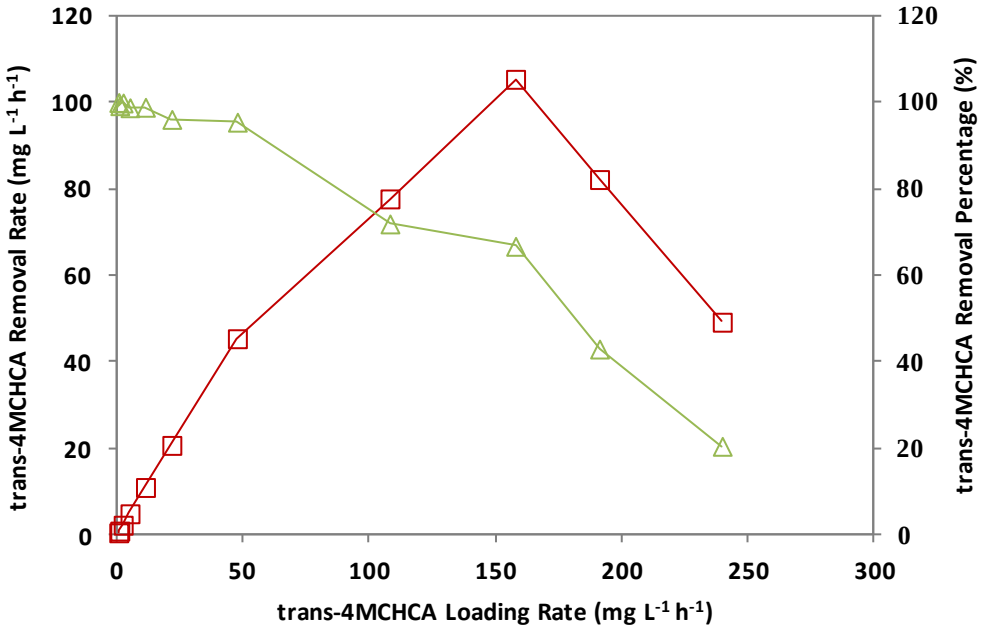


Figure 4.7. Removal rate and removal percentage of trans-4MCHCA as a function of trans-4MCHCA loading rate. □ removal rate of trans-4MCHCA; Δ removal percentage of trans-4MCHCA.

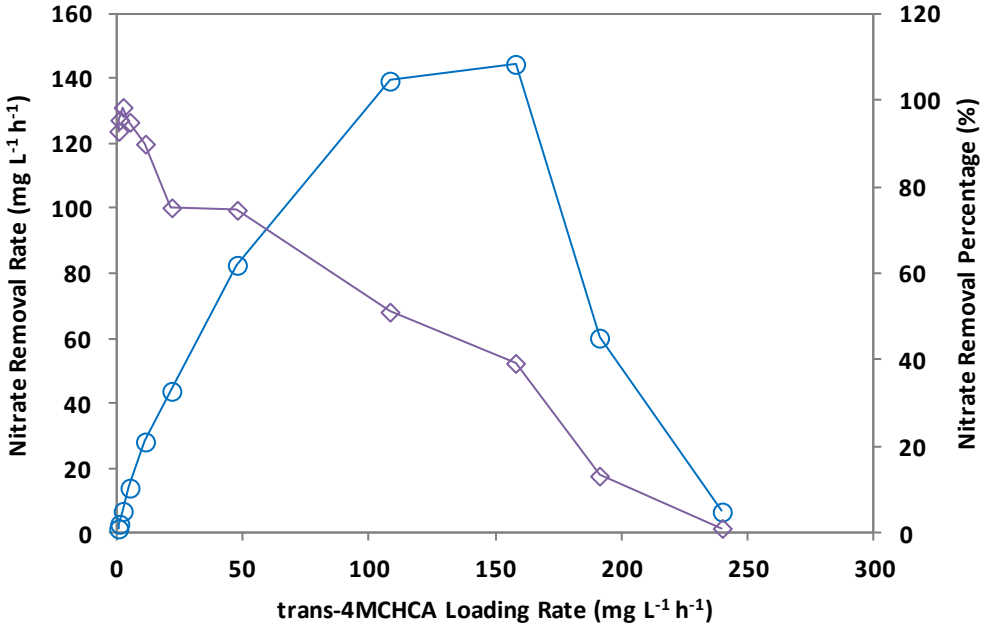


Figure 4.8. Removal rate and removal percentage of nitrate as a function of trans-4MCHCA loading rate. ○ removal rate of nitrate; ◊ removal percentage of nitrate.

At the same loading rate (157.8 mg L^{-1} h^{-1}), the removal percentage of trans-4MCHCA and nitrate achieved were 66.8% and 39.3%, respectively. For dilution rate of 0.051 h^{-1} (corresponding trans-4MCHCA loading rate: 11.1 mg L^{-1} h^{-1}), where the maximum biomass concentration achieved (178.3 mg L^{-1}), the removal percentage of trans-4MCHCA and nitrate were 98.9% and 90.0%, respectively. However, as the dilution rate was increased to 0.187 h^{-1} (trans-4MCHCA loading rate: 47.6 mg L^{-1} h^{-1}), the biomass concentration slightly decreased and reached 109.3 mg L^{-1} with the percentage conversion of trans-4MCHCA decreased drastically from 95.4% to 71.9%, and then further decreased to percentage of 20.5% at the end of the experiment. The biomass concentration measured at 20.5% trans-4MCHCA removal percentage was 3.0 mg L^{-1}.

4.3. Continuous Biodegradation of trans-4MCHCA in Biofilm Reactor

The effects of volumetric loading rate and concentration of trans-4MCHCA were evaluated in the biofilm reactor. The performance of the biofilm reactor was assessed in similar way to CSTR. Removal percentage and biodegradation rates for trans-4MCHCA and nitrate were calculated to evaluate the performance of biofilm. Initially, biofilm reactor was fed with 50.4 ± 0.2 mg L^{-1} trans-4MCHCA and 293.3 ± 22.8 mg L^{-1} nitrate at a flow rate of 0.5 mL h^{-1} (corresponding trans-4MCHCA loading rate: 1.0 mg L^{-1} h^{-1} or residence time: 50.0 h). The flow rate (loading rate) was then increased stepwise. Figure 4.9 illustrates the steady state concentrations of trans-4MCHCA and nitrate in the effluent observed at various loading rate ranging from 1.0 to 478.1 mg L^{-1} h^{-1} (corresponding residence times: 50.0 to 0.1 h). Trans-4MCHCA was the limiting substrate thus loading rate of trans-4MCHCA was used to present the performance of the reactor. In a biofilm system, microorganisms are attached to the matrix provided for this purpose; therefore applying high flow rate would not result in cell washout.

Figure 4.9 shows that at 1.0 mg L^{-1} h^{-1} loading rate, the residual trans-4MCHCA and nitrate concentration in biofilm reactor were 0.6 mg L^{-1} and 96.7 mg L^{-1}, respectively. The residual trans-4MCHCA and nitrate concentrations then increased gradually as the loading rate of trans-4MCHCA was increased. At the highest loading rate tested in the biofilm reactor (478.1 mg L^{-1} h^{-1}), the residual trans-4MCHCA and nitrate concentrations were 15.2 mg L^{-1} and 217.0 mg L^{-1}, respectively, with the corresponding removal percentage of trans-4MCHCA and nitrate being 69.7% and 26.2%, respectively. There was no or little production of nitrite in the system. Due to the attached nature of biomass, data for biomass concentration is not provided.

Figure 4.10 and 4.11 illustrate the linear increase in removal rate of trans-4MCHCA and nitrate as loading rate of trans-4MCHCA was increased. The biofilm system reached the maximum removal rate of trans-4MCHCA of 398.1 mg L^{-1} h^{-1}, accompanied by maximum removal rate of nitrate (816.1 mg L^{-1} h^{-1}) at corresponding trans-4MCHCA loading rate of 435.8 mg L^{-1} h^{-1} (residence time: 0.1 h). The removal percentage of trans-4MCHCA under maximum removal rate of both trans-4MCHCA and nitrate (trans-4MCHCA loading rate: 435.8 mg L^{-1} h^{-1}), was 91.4%, while the removal percentage of nitrate was 31.1%. The removal percentage of trans-4MCHCA stayed in the range of 88 – 98% for loading rates up to 435.8 mg L^{-1} h^{-1}. As the loading rate was further increased to 478.1 mg L^{-1} h^{-1}, the trans-4MCHCA removal percentage decreased drastically from 91.4% to 69.7%. The removal percentage of nitrate decreased from 90.5% at a loading rate of 6.1 mg L^{-1} h^{-1} to 48.9% at the loading rate of 9.2 mg L^{-1} h^{-1}, then the nitrate removal percentage stayed in the range of 25 – 35%. When the trans-4MCHCA loading rate was further increased to 478.1 mg L^{-1} h^{-1} at the end of the experiment, the removal percentage of nitrate was only 26.2% (shown in Figure 4.11).

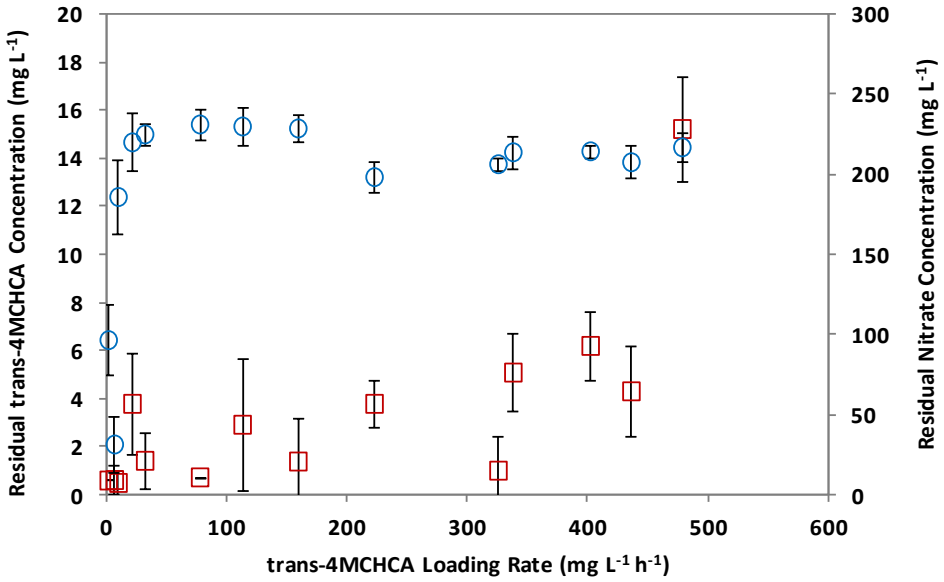


Figure 4.9. Effect of trans-4MCHCA loading rate on trans-4MCHCA and nitrate residual concentrations in the biofilm system. (□ trans-4MCHCA concentration; ○ nitrate concentration in biofilm reactor). Values reported are the average from multiple samplings of the reactor over an extended period equal to 3-5 residence times after the establishment of steady state. Error bars represent the standard deviations, which might not be visible in some cases.

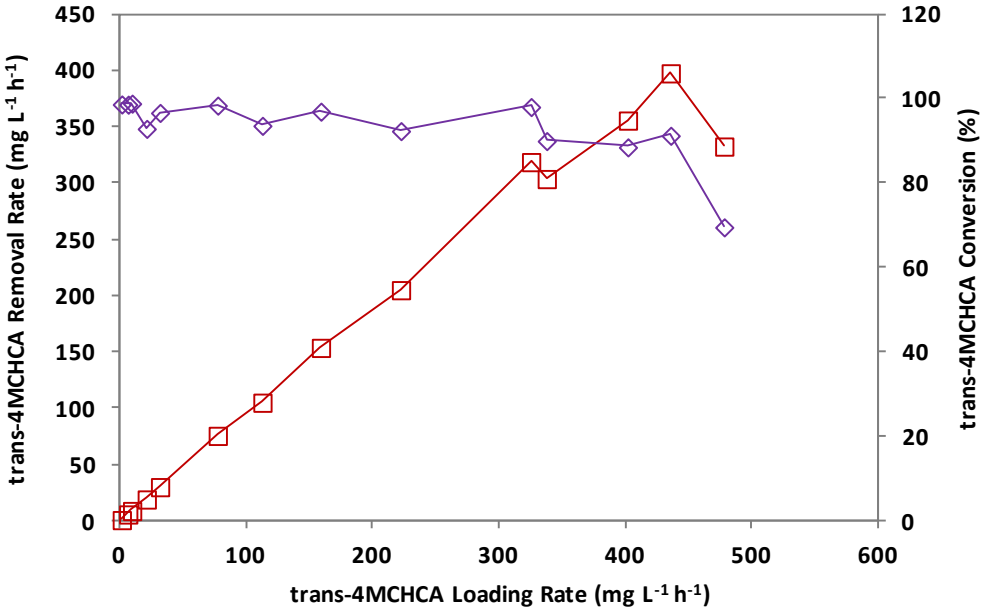


Figure 4.10. Removal rate and removal percentage of trans-4MCHCA (feed concentration of 50.3 mg L^{-1}) as a function of trans-4MCHCA loading rate in the biofilm reactor. □ removal rate of trans-4MCHCA; ◊ removal percentage of trans-4MCHCA.

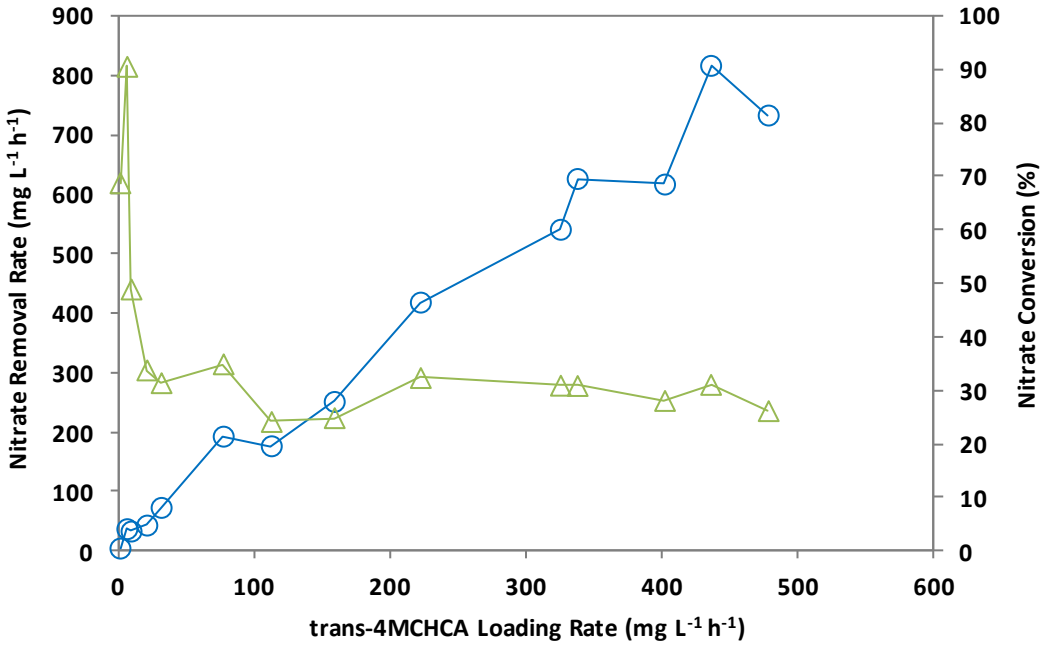


Figure 4.11. Removal rate of nitrate (feed concentration of 316.1 mg L^{-1}) and nitrate removal percentage as a function of trans-4MCHCA loading rate in the biofilm reactor. (○ removal rate of nitrate; Δ removal percentage of nitrate).

Upon completion of this part of experiments, where loading rate was increased with increase of flow rate, the biofilm reactor was continuously run to study the effect of different concentrations of trans-4MCHCA and nitrate on biodegradation and nitrate removal rates when the flow rates were maintained at an approximate level around 110 mL h^{-1}. Feed concentration of trans-4MCHCA was increased from 50.4 ± 0.2 mg L^{-1} to 93.8 ± 3.6, 237.7 ± 16.8, and 592.7 ± 9.9 mg L^{-1} accompanied by adjustment in corresponding nitrate concentration from 293.3 ± 2.8 mg L^{-1} to 283.4 ± 0.9, 619.4 ± 3.5, and 912.7 ± 17.6 mg L^{-1}. Figure 4.12 shows that increase in loading rate of trans-4MCHCA increased the residual concentration of trans-4MCHCA in the biofilm reactor. For example, at the feed concentration of 50.4 ± 0.2 mg L^{-1} trans-4MCHCA and 293.3 ± 2.8 mg L^{-1} nitrate, the trans-4MCHCA residual concentration and nitrate were 3.8 ± 0.9 mg L^{-1} and 218.3 ± 44.0 mg L^{-1}, respectively. When the feed concentration was increased to 93.8 ± 3.6,

237.7 ± 16.8, and 592.7 ± 9.9 mg L^{-1}, the residual concentration of trans-4MCHCA in biofilm reactor increased to 0.4 ± 0.3, 9.3 ± 2.6, and 131.8 ± 3.9 mg L^{-1}, respectively. However, the nitrate residual concentration profile did not have a specific pattern due to the excessive amount of nitrate provided to the reactor to prevent nitrate being the limited substrate. There was no or little nitrite was present in the biofilm reactor for all tested feed concentrations.

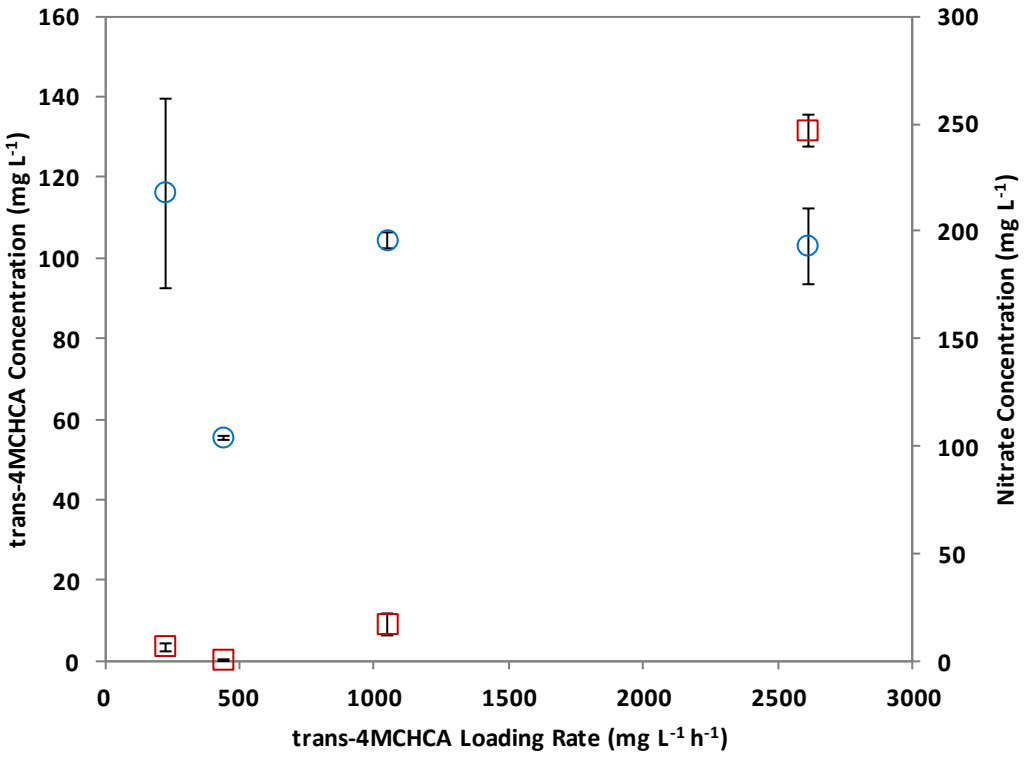


Figure 4.12. Residual concentrations of trans-4MCHCA and nitrate in the effluent of biofilm fed with various concentrations of trans-4MCHCA and nitrate. □ residual concentration of trans-4MCHCA; ○ residual concentration of nitrate.

The removal rate of trans-4MCHCA was calculated and shown in Figure 4.13. As seen, the removal rate of trans-4MCHCA increased as loading rate was increased. At the lowest trans-4MCHCA feed concentration of 50.4 ± 0.2 mg L^{-1} (corresponding trans-4MCHCA loading rate of 222.3 mg L^{-1} h^{-1}), the trans-4MCHCA removal rate achieved was 205.6 mg L^{-1} h^{-1} with trans-

4MCHCA removal percentage calculated to be 92.5%, while the nitrate removal rate was 330.9 mg L^{-1} h^{-1} with removal percentage of nitrate being 25.6%. When the trans-4MCHCA feed concentration was increased from 50.4 ± 0.2 mg L^{-1} to 93.8 ± 3.6 mg L^{-1}, the reactor was provided with the nitrate feed concentration at approximate concentration of 283.4 ± 8.1 mg L^{-1}, resulting in increase of removal rate of nitrate to 836.8 mg L^{-1} h^{-1} with its removal percentage of 63.2%.

As previously mentioned, higher trans-4MCHCA loading rate (achieved by increase in flow rate) increased the removal rate of trans-4MCHCA. A similar pattern was observed when loading rate was increased through increases in feed concentration. When feed concentration of trans-4MCHCA was increased from 50.4 ± 0.2 mg L^{-1} to 93.8 ± 3.6, 237.7 ± 16.8, and 592.7 ± 9.9 mg L^{-1}, the trans-4MCHCA removal rates increased to 436.2, 1,007.6, and finally reached the highest value of 2,028.1 mg L^{-1} h^{-1}. The corresponding removal percentages of trans-4MCHCA were 99.6%, 96.1%, and 77.8%, respectively. These results demonstrated that high concentration of substrate did not inhibit the biodegradation process. The highest removal rate of nitrate of 3,164.7 mg L^{-1} h^{-1}, with nitrate removal percentage of 78.8%, was achieved at hydraulic retention time of 0.23 h (corresponding trans-4MCHCA loading rate: 2,607.9 mg L^{-1} h^{-1}) when the reactor was fed with the highest concentration of trans-4MCHCA and nitrate.

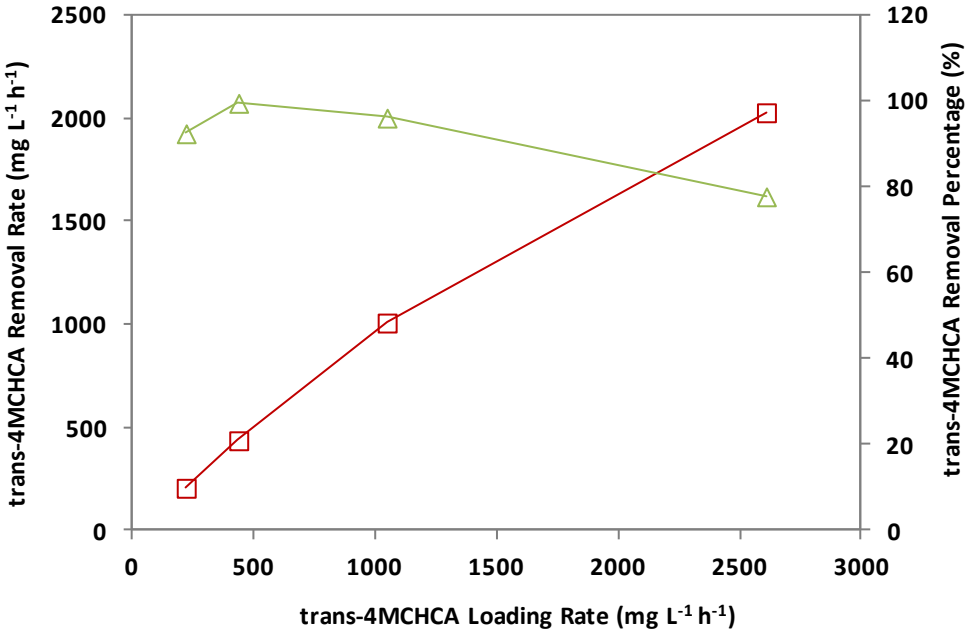


Figure 4.13. Removal rate and removal percentage of trans-4MCHCA as a function of its loading rate in the biofilm reactor fed with various concentrations of trans-4MCHCA. (□ removal rate of trans-4MCHCA; Δ removal percentage of trans-4MCHCA).

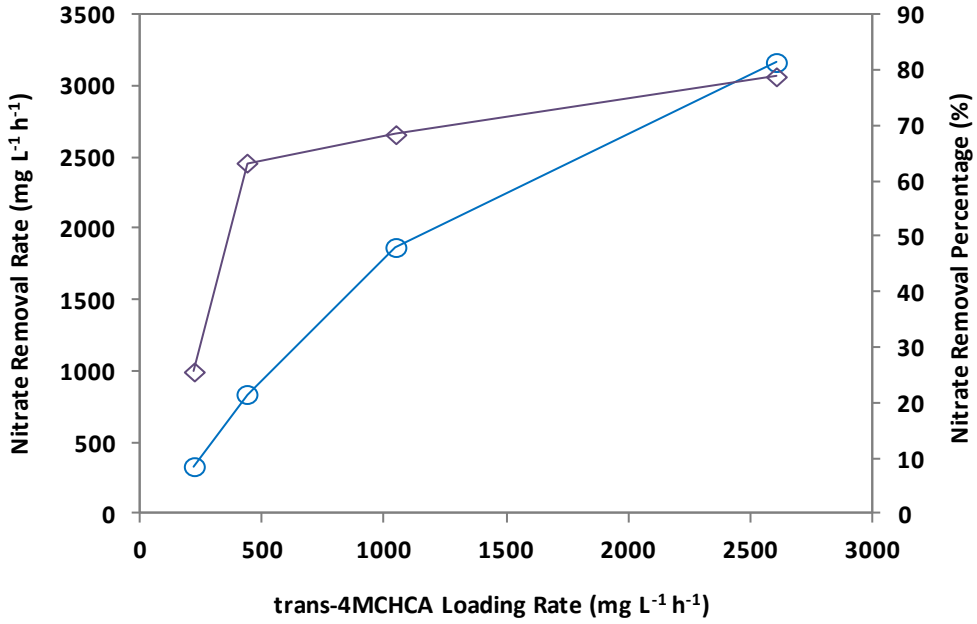


Figure 4.14. Removal rate and removal percentage of nitrate as a function of trans-4MCHCA loading rate in the biofilm reactor fed with various concentrations of trans-4MCHCA. (◊ removal rate of nitrate; ○ removal percentage of nitrate).

4.4. Comparison of Anaerobic Biodegradation in the CSTR and Biofilm Reactor

In this section, the performance of CSTR and biofilm reactor operated under anaerobic conditions are compared using the removal rates of trans-4MCHCA and nitrate. The removal rate profile of trans-4MCHCA in CSTR operated with trans-4MCHCA (feed concentration of 250 mg L^{-1} and nitrate concentration of 620 mg L^{-1}) is presented in Figure 4.15 (A), and the same profile in biofilm reactor operated with trans-4MCHCA feed concentrations ranged from 50 to 500 mg L^{-1} and nitrate concentrations in the range 310 to 930 mg L^{-1} is presented in Figure 4.15 (B). In CSTR, the maximum removal rate of trans-4MCHCA was 105.4 mg L^{-1} h^{-1} and obtained at a loading rate of 157.8 mg L^{-1} h^{-1}. On the other hand, in the biofilm system, the maximum trans-4MCHCA removal rate of 1,007.6 mg L^{-1} h^{-1} achieved at a trans-MCHCA loading rate of 1,048.4 mg L^{-1} h^{-1}. The maximum removal rate of trans-4MCHCA in biofilm reactor appeared to be approximately ten times higher than that in CSTR with freely suspended cells, when the reactors were run with similar feeds (same trans-4MCHCA and nitrate concentrations).

Biofilm reactor could be operated with much higher loading rate of trans-4MCHCA (2,607.9 mg L^{-1} h^{-1} vs 239.8 mg L^{-1} h^{-1}) and much shorter residence time (0.2 h vs 1.0 h) compared to CSTR. Figure 4.16 shows that the performances of these two bioreactors with respect to nitrate removal rate were similar to that observed for trans-4MCHCA. For example, at trans-4MCHCA loading rate of 157.8 mg L^{-1} h^{-1}, CSTR reached the maximum nitrate removal rate of 144.5 mg L^{-1} h^{-1}, while in the biofilm reactor, it was possible to increase in loading rate up to 2,607.9 mg L^{-1} h^{-1} and achieve a higher nitrate removal rate of 3,164.7 mg L^{-1} h^{-1}.

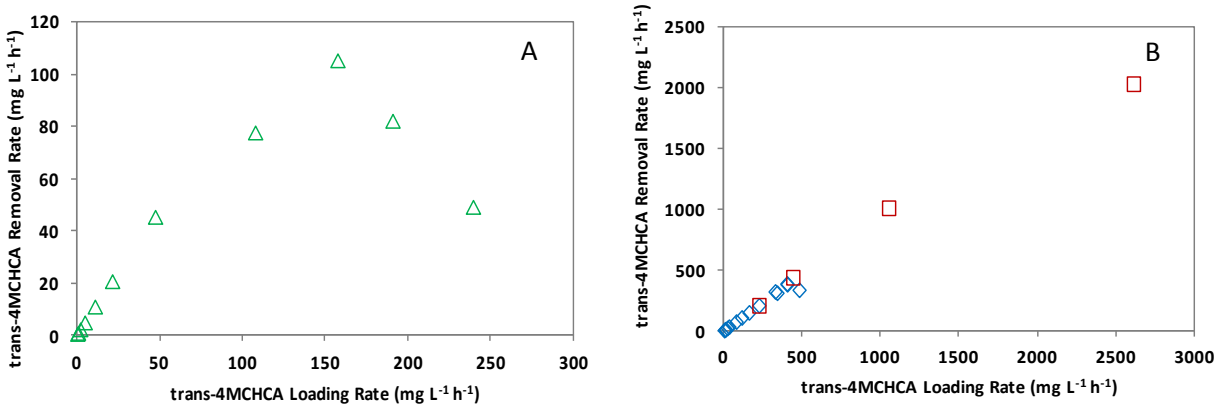


Figure 4.15. Comparison of trans-4MCHCA removal rates in CSTR and biofilm reactor. (A) Δ CSTR operating at initial trans-4MCHCA concentration of 250 mg L^{-1} h^{-1} and 620 mg L^{-1} of nitrate; (B) ◊ Biofilm reactor operating at initial trans-4MCHCA concentration of 50 mg L^{-1} h^{-1} and 310 mg L^{-1} of nitrate under various feed flow rate; □ Biofilm reactor operated at a constant feed flow rate (~110 mL h^{-1}) with varied feed concentration of trans-4MCHCA and nitrate.

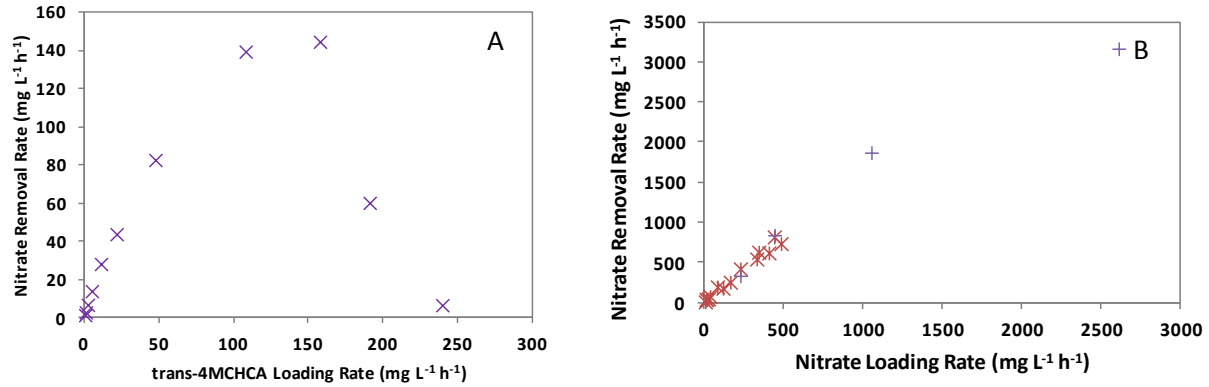


Figure 4.16. Comparison of nitrate removal rates of two different reactors. (A) × CSTR operating at initial trans-4MCHCA concentration of 250 mg L^{-1} h^{-1} and 620 mg L^{-1} of nitrate; (B) ж Biofilm reactor operating at initial trans-4MCHCA concentration of 50 mg L^{-1} h^{-1} and 310 mg L^{-1} of nitrate under various feed flow rate; + Biofilm reactor operating at a constant feed flow rate (~110 mL h^{-1}) with varied feed concentration of trans-4MCHCA and nitrate.

4.5. Comparison of Aerobic and Anaerobic Biodegradation Processes

4.5.1. Comparison of Aerobic and Anaerobic Biodegradation in Batch System

Table 4.3 compares the biodegradation rates of trans-4MCHCA obtained at different initial concentrations and temperatures under anaerobic conditions with those obtained under aerobic conditions as part of an earlier work (Paslawski *et al.*, 2009). Data in Table 4.3 shows that the rates obtained in current study were close to those obtained under aerobic condition, which had rates ranging from 1.1 to 5.6 mg L^{-1} h^{-1}. The result also shows that effects of NA concentration and temperature on anaerobic biodegradation were similar to those observed under aerobic condition. In other words increase in initial concentration of trans-4MCHCA to 250 mg L^{-1} led to higher biodegradation rates, though in case of aerobic biodegradation increase of biodegradation rate occurred as a result of concentration increases up to 500 mg L^{-1}.

Table 4.3. Comparison of biodegradation rates of trans-4MCHCA obtained at various initial concentrations at room temperature (24 ± 2°C) under aerobic (Paslawski *et al.*, 2009) and anaerobic conditions (present work)

trans-4MCHCA Concentration (mg L^{-1})	Aerobic Biodegradation Rate (mg L^{-1} h^{-1}) (Paslawski *et al.*, 2009)	Anaerobic Biodegradation Rate (mg L^{-1} h^{-1}) (present work)
100	1.1 ± 0.1	1.3
250	1.9 ± 0.1	4.7
500	4.8 ± 0.3	5.1 ± 0.3
750	5.6 ± 0.6	5.3 ± 0.1

Aerobic and anaerobic biodegradation rates of trans-4MHCA at different temperatures are compared in Table 4.4. As seen in this table, the optimum temperature for biodegradation of trans-4MCHCA under both aerobic and aerobic conditions was found to be in the range 20 to 24°C (room temperature) with the highest biodegradation rate achieved was approximately 4.7 to 4.8 mg L^{-1} h^{-1}.

Table 4.4. Comparison of biodegradation rates of trans-4MCHCA obtained at various temperatures at initial concentration of trans-4MCHCA of 250 mg L^{-1}, under aerobic (Paslawski *et al.*, 2009) and anaerobic conditions (present work)

Temperature (°C)	Aerobic Biodegradation Rate (Paslawski *et al.*, 2009) (mg L^{-1} h^{-1})	Temperature (°C)	Anaerobic Biodegradation Rate (present work) (mg L^{-1} h^{-1})
12	1.2 ± 0.1	10	0.2 ±0.1
16	0.9 ± 0.0	15	0.6 ± 0.1
		20	4.1 ± 0.3
23	4.8 ± 0.3	24 ± 2	4.7
30	1.1 ± 0.1	30	2.8 ± 0.2
37	3.6 ± 0.3	35	2.9

As seen in Table 4.4 the influence of temperature on biodegradation under both conditions was in agreement in which decrease or increase of temperature from the optimum value caused a decrease in biodegradation rate. At temperature below room temperature (10° to 16°C), there was a decrease in biodegradation rate of trans-4MCHCA to values in the range of 0.2 to 1.2 mg L^{-1} h^{-1}, while at temperature above room temperature (30° to 35°C), the biodegradation rate was decreased to values in the range of 1.1 to 3.6 mg L^{-1} h^{-1}. The comparison result suggested that biodegradation of trans-4MCHCA could be achieved under aerobic or anaerobic condition, with room temperature was identified as the optimum temperature for microbial activity.

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4.5.2. Comparison of Aerobic and Anaerobic Biodegradations in CSTR

Aerobic CSTR used in an earlier work was operated with 500 mg L^{-1} trans-4MCHCA in the feed and had a working volume of 550 mL (Paslawski *et al.*, 2009), while anaerobic CSTR used in the current work was operated with 250 mg L^{-1} of trans-4MCHCA in the feed and had a working volume of 200 mL. Therefore, comparison of the performances was carried out using volumetric loading and removal rates so these differences can be incorporated. Variation of removal rate of

trans-4MCHCA as a function of its loading rate for CSTRs operated under aerobic and anaerobic conditions are compared in Figure 4.17. The biodegradation profile showed a similar pattern under both aerobic and anaerobic conditions. When the loading rate of trans-4MCHCA was increased, there was an increase in trans-4MCHCA removal rate; the system reached the maximum rate then the removal rate of trans-4MCHCA started to decline due to significant decrease in biomass concentration and eventual washout. The highest trans-4MCHCA removal rate obtained in anaerobic CSTR was 105.4 mg L^{-1} h^{-1} at a trans-4MCHCA loading rate of 154.8 mg L^{-1} h^{-1}, while the highest trans-4MCHCA removal rate achieved in aerobic CSTR was reported to be 9.6 mg L^{-1} h^{-1} at a trans-4MCHCA loading rate of 14.2 mg L^{-1} h^{-1}. Using the corresponding biomass concentrations, the maximum specific removal rate in anaerobic and aerobic CSTR was calculated and compared (maximum specific trans-4MCHCA removal rate was calculated by dividing the maximum trans-4MCHCA removal rate by the corresponding biomass concentration). Anaerobic CSTR had maximum specific trans-4MCHCA removal rate of 2.93 mg substrate mg $biomass^{-1}$ h^{-1}, which is higher than that observed in the aerobic CSTR (0.15 mg substrate mg $biomass^{-1}$ h^{-1}). The comparison result showed that CSTR operated under anaerobic condition had a specific removal rate which was at least 20 fold higher than that obtained under aerobic condition. This resulted in higher removal rate of tran-4MCHCA in anaerobic CSTR, which was eleven times higher than the aerobic CSTR.

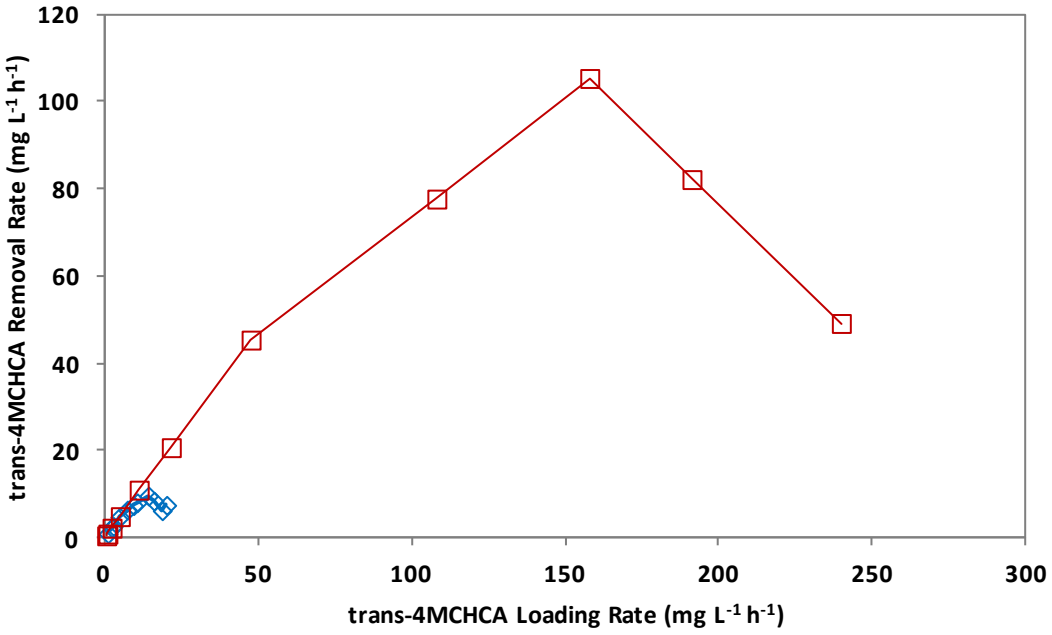

Figure 4.17. Removal rate of trans-4MCHCA as a function of its loading rate in continuous CSTR operated under aerobic and anaerobic conditions. □ Anaerobic CSTR fed with 232.5 ± 22.9 mg L^{-1} trans-4MCHCA; ◊ Aerobic CSTR fed with 500 mg L^{-1} trans-4MCHCA in (data was taken from Paslawski *et al.*, 2009).

4.5.3. Comparison of Aerobic and Anaerobic Biodegradations in Biofilm System

The performance of the continuous biofilm reactors operated under aerobic and anaerobic conditions are compared in this section. The anaerobic biofilm reactor was initially fed with 50 mg L^{-1} of trans-4MCHCA and 293 mg L^{-1} nitrate. After completion of this part of study where loading rate was changed by variation of flow rates with same initial feed concentration, the effect of different feed concentrations was tested. Trans-4MCHCA at concentrations of 50.0, 93.8, 237.7, and 592.7 mg L^{-1} were included in the feed to increase the loading rate of trans-4MCHCA (in this part, flow rate was kept constant). The aerobic biofilm reactor used in an earlier work (Paslawski *et al.*, 2009) had been fed with 500 mg L^{-1} of trans-4MCHCA under

various flow rates. Figure 4.18 compares the results obtained in the biofilm reactors operated under aerobic and anaerobic conditions. In either case, removal rate of trans-MCHCA increased linearly with the increase in loading rate. However, in the aerobic system, removal rate of trans-4MCHCA increased, reached its maximum rate, and then started to decrease, while in anaerobic system, as the loading rate increased, the removal rate of trans-4MCHCA was further increased, indicating that anaerobic biofilm reactor had the potential to be operated at much higher loading rates when compared with the aerobic biofilm reactor. The highest removal rate obtained in the anaerobic biofilm reactor was 2,028.1 mg L^{-1} h^{-1} at a trans-4MCHCA loading rate of 2,607.9 mg L^{-1} h^{-1}, while the highest removal rate achieved in aerobic biofilm reactor was 924.4 mg L^{-1} h^{-1} at a trans-4MCHCA loading rate of 924.4 mg L^{-1} h^{-1} (Paslawski *et al.,* 2009). The comparison result shows that operated under anaerobic condition, biofilm reactor had almost 2.8 times higher removal rate than the bioreactor which operated under aerobic condition. Due to difficulty in measurement of biomass hold-up in the anaerobic biofilm reactor the comparison of the specific removal rates was not conducted.

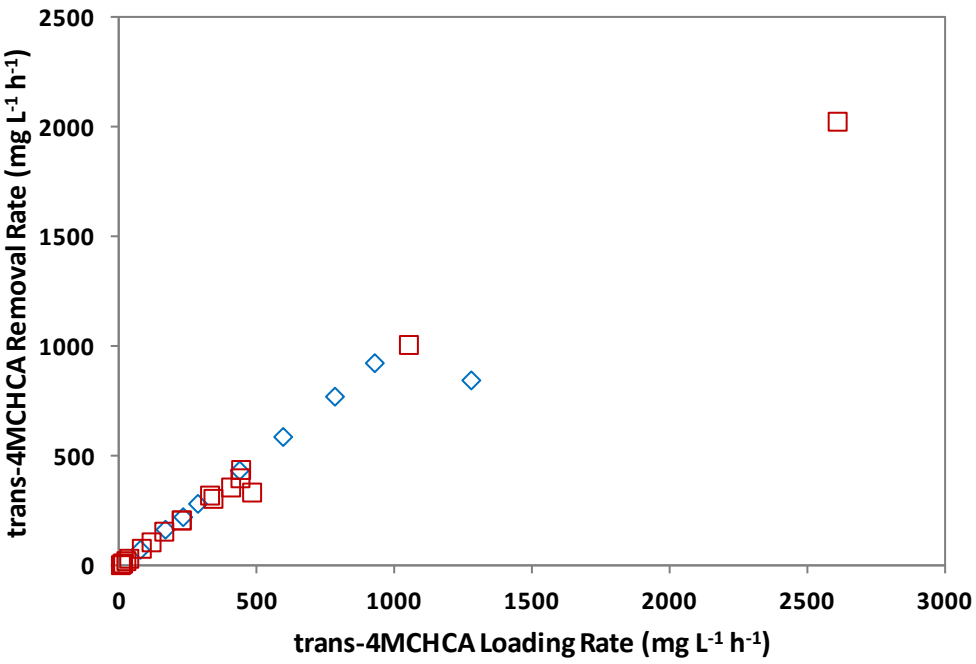

Figure 4.18. Removal rate of trans-4MCHCA as a function of its loading rate in continuous biofilm reactors operated under aerobic and anaerobic conditions. □ Anaerobic biofilm reactor fed with 50.0, 93.8, 237.7, and 592.7 mg trans-4MCHCA L^{-1} (current study); ◊ Aerobic biofilm reactor fed with 500 mg trans-4MCHCA L^{-1} (Paslawski *et al.*, 2009).

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Bioremediation is considered as one of the most effective and economical approach for treating NAs in OSPW (Khan *et al.*, 2013). Previous studies focused on aerobic biodegradation of three different model naphthenic acids: octanoic acid, trans-4MCHCA, mixture of cis- and trans-4MCHAA (Paslawski *et al.*, 2009; Huang *et al.*, 2012; D'souza, 2012). The present work investigated the kinetics and parameters that affect anaerobic biodegradation of a surrogate naphthenic acid, trans-4MCHCA, and reduction of nitrate. Evaluating anaerobic biodegradation is necessary to verify the possibility of this option as an alternative for treating NAs with the advantage of eliminating the cost of aeration associated with aerobic system and the potential for in-situ treatment of OSPW in anaerobic stabilization ponds.

The biodegradation of trans-4MCHCA under denitrifying condition was studied in batch, continuous stirred tank, and biofilm reactors. Results of batch study on initial concentration of trans-4MCHCA in the range 100 to 750 mg L^{-1} demonstrated: 1)- that increase in initial concentration of trans-4MCHCA required higher concentration of nitrate (electron acceptor), indicating the coupling of biodegradation and denitrification processes; 2)- the increase in initial concentration of trans-4MCHCA from 100 to 250 mg L^{-1} led to higher rates but further increase in initial concentration did not have a marked effect. Moreover, batch experiments demonstrated that although effective biodegradation was achieved for all tested temperatures (10° to 35°C), the optimum temperature was in the range of 20 - 24°C.

Biodegradation of trans-4MCHCA under anaerobic condition was also investigated in the CSTR. In this case as the loading rate of trans-4MCHCA was increased, an increase in removal rate of both trans-4MCHCA and nitrate was observed. Rates were decreased as the system approached the cell washout. The maximum removal rate of biodegradation and nitrate achieved at loading rate of 157.8 mg L^{-1} h^{-1} in CSTR were 105.4 mg L^{-1} h^{-1} and 144.5 mg L^{-1} h^{-1}, respectively. A similar dependency between the loading and removal rates was also observed in the biofilm reactor. The maximum removal rate of trans-4MCHCA and nitrate in the biofilm reactor operated at room temperature (24 ± 2ºC) were 2,028.1 mg L^{-1} h^{-1} and 3,164.7 mg L^{-1} h^{-1}, respectively and obtained at trans-4MCHCA loading rate of 2,607.9 mg L^{-1} h^{-1}. Based on these findings, biofilm system was shown to outperform CSTR with ability to enhance biodegradation rate up to nineteen times higher than CSTR rate due to capability of providing a high biomass hold-up and decoupling of hydraulic residence time from the biomass residence time.

Comparison between results obtained in the aerobic batch system by Paslawski *et al.* (2008) and the current results was also conducted. It appears that this is the first study in which performances of the aerobic and anaerobic system for biodegradation of a model naphthenic acid are compared. Aerobic and anaerobic biodegradation in batch systems showed similar profile where increase in initial concentration of naphthenic acid increased the biodegradation rate of trans-4MCHCA. The result also showed that anaerobic biodegradation rates were close to those obtained under aerobic conditions. As far as the effect of temperature is concerned room temperature (20 - 24 ºC) was identified as optimum temperature regardless of mode of biodegradation.

By contrast, under continuous mode of operation (CSTR and biofilm reactors), anaerobic biodegradation was much faster than its aerobic counterpart. For instance the maximum anaerobic removal rate of trans-4MCHCA in the CSTR was 105.4 mg L^{-1} h^{-1}, while the highest removal rate achieved in the aerobic CSTR was 9.6 mg L^{-1} h^{-1}. Similarly, anaerobic biofilm reactor achieved a higher maximum removal rate of 2,028.1 mg L^{-1} h^{-1} compared to a 924.4 mg L^{-1} h^{-1} removal rate in the aerobic biofilm reactor. The overall finding indicated that biodegradation of trans-4MCHCA can be achieved effectively under anaerobic condition with the rates markedly higher than those for aerobic system.

5.2. Recommendations

Further work with NAs with more complex structure and combination of NAs in a mixture (co-biodegradation) should be carried out to unfold the full potential of anaerobic system. Study of anaerobic biodegradation using nitrite as electron acceptor NAs is also important since nitrite is the intermediate product of nitrate reduction during the denitrification process. Also, utilization of of other electron acceptor such as sulfate, iron, or manganese for anaerobic biodegradation of NAs should be investigated. Another important aspect of NA anaerobic biodegradation is the reaction and metabolic pathways which are involved. A detailed study on this aspect would be essential. Finally, application of anaerobic biodegradation in treatment of commercial mixture of NAs and OSPW would be critical in development of a proper treatment technology.

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APPENDIX A

Biomass Calibration Curve

Calibration curve for biomass concentration was developed by direct measurement of the optical density (OD) of the samples taken from serum bottles at a wavelength of 620 nm (Paslawski, 2008). An ultraviolet (UV) spectrophotometer (Mini Shimadzu, Model 1240) was used to determine of OD. The linear calibration curve (Figure A.1) was required to convert the OD to biomass dry weight per mL.

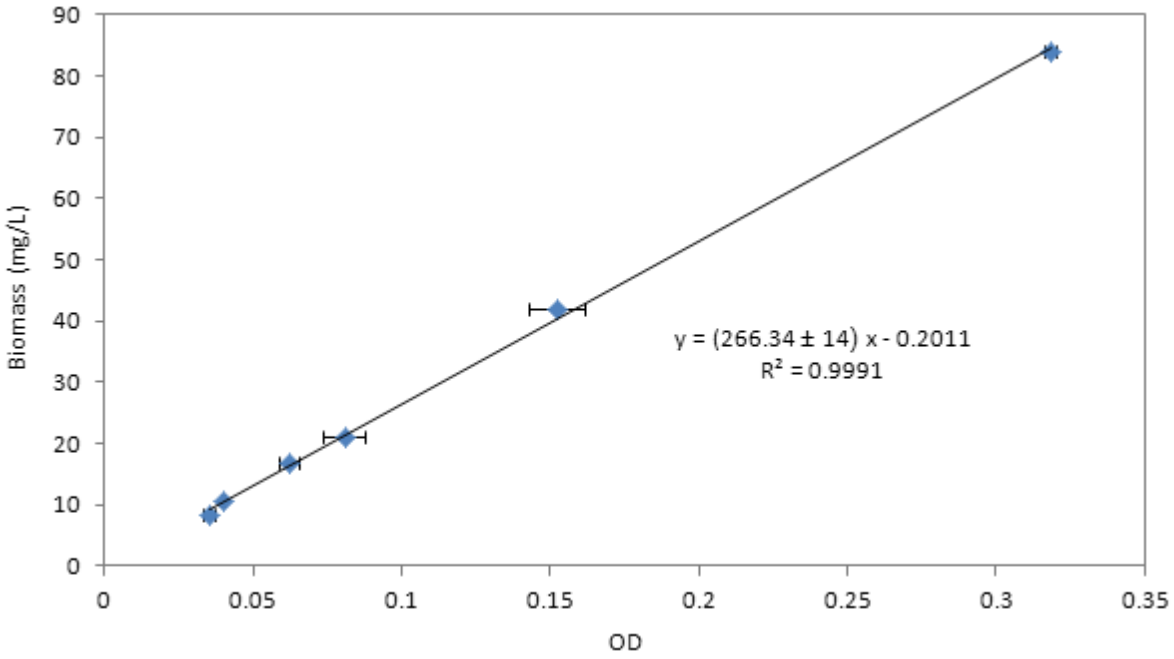

Figure A.1. Biomass calibration curve. Error bars represent standard deviations in optical density readings.

Ion Chromatography (IC) Calibration Curve

A calibration curve for Ion Chromatography (IC) was generated using six standard solutions (5, 10, 20, 30, and 50 mg L^{-1}) in the sterile modified McKinney's medium. Calibration was carried out regularly to ensure the accuracy of experimental results. Dionex ICS-2500 IC was used for the measurement of nitrate and nitrite concentration. The representative calibration curves for nitrate and nitrite are presented in Figure A.2 and A.3. IC showed nitrite and nitrate peaks at retention times of 4.8 and 7.0 min, respectively.

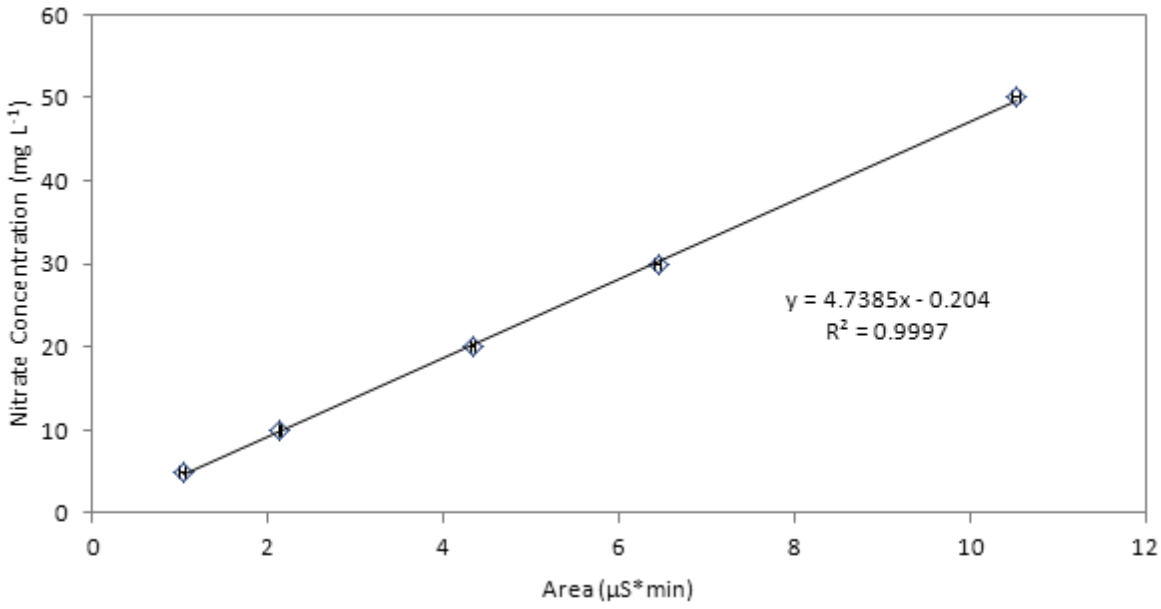

Figure A.2. Calibration curve developed for various nitrate concentrations. Error bars represent standard deviations in multiple nitrate concentration readings.

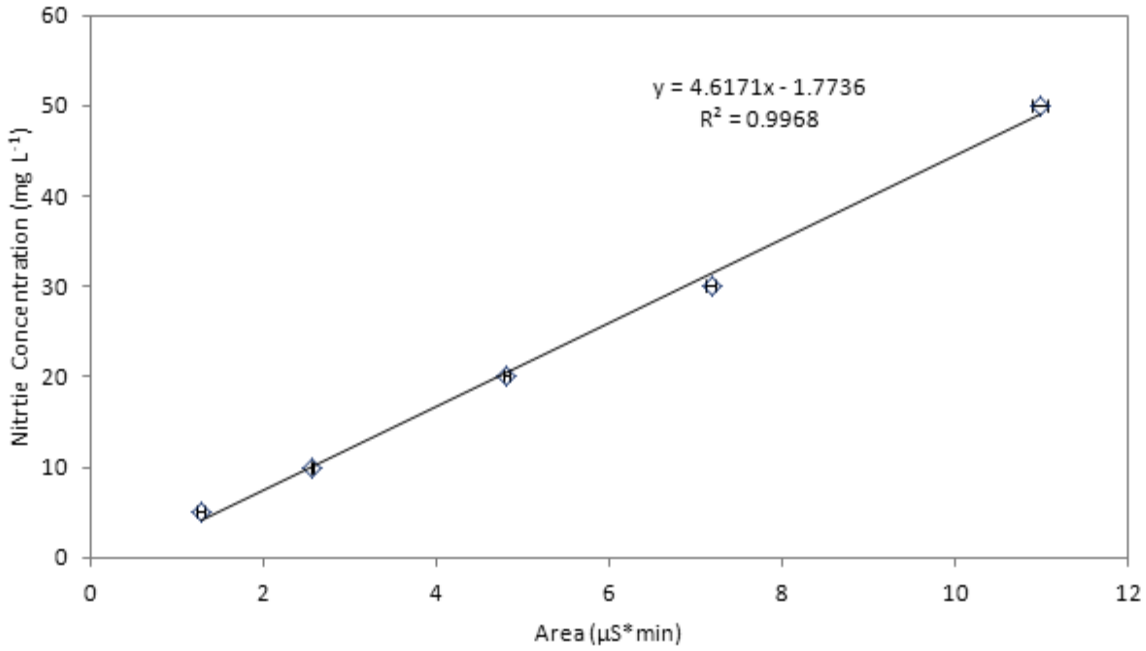

Figure A.3. Calibration curve developed for various nitrite concentrations. Error bars represent standard deviations in multiple nitrite concentration readings.

Gas Chromatography (GC) Calibration Curve

A linear calibration curve for Gas Chromatography (GC) was generated using six standard solutions (10, 25, 50, 75, 100, and 125 mg L^{-1}) in the sterile modified McKinney's medium. Calibration was carried out regularly to ensure the accuracy of experimental results. GC-FID with split/splitless injector was used for the measurement of trans-4MCHCA concentration. The representative calibration curve for trans-4MCHCA is presented in Figure A.4. GC showed trans-4MCHCA peak at retention times of 3.6 ± 0.2 min.

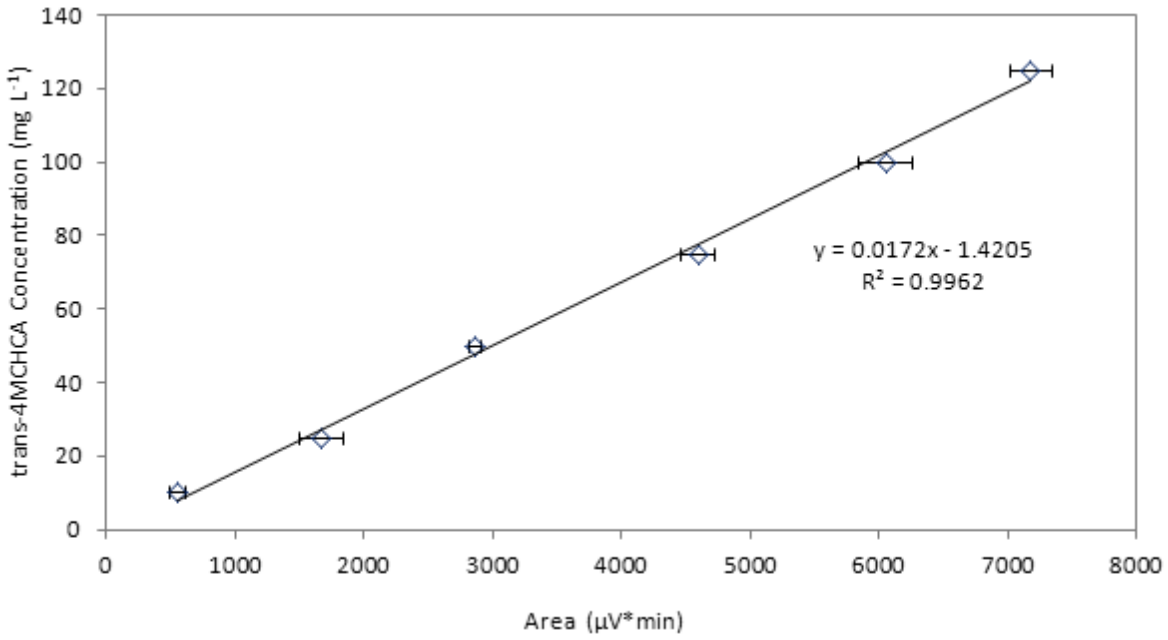

Figure A.4. Calibration curve developed from various trans-4MCHCA concentrations. Error bars represent standard deviations in trans-4MCHCA concentration readings.

APPENDIX B

Sample of Ion Chromatogram

Figure B.1 shows the elution time of both nitrate and nitrite ion using Dionex ICS-2500 ion chromatography. The elution time of different ions was noted 4.8 minutes for nitrite and 7.0 minutes for nitrate, respectively.

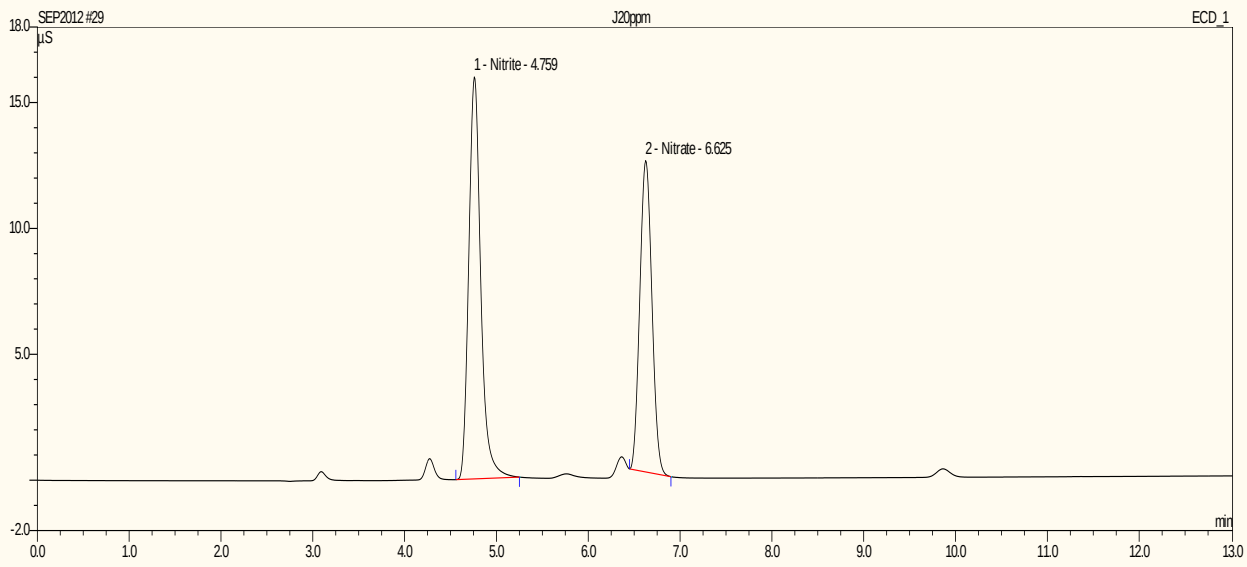

Figure B.1. The representative Dionex ICS-2500 IC chromatogram of nitrate and nitrite.

Samples of Gas Chromatogram

Figure B.3 shows the elution time of trans-4MCHCA using gas chromatography. The elution time of trans-4MCHCA was 3.6 ± 0.2 min.

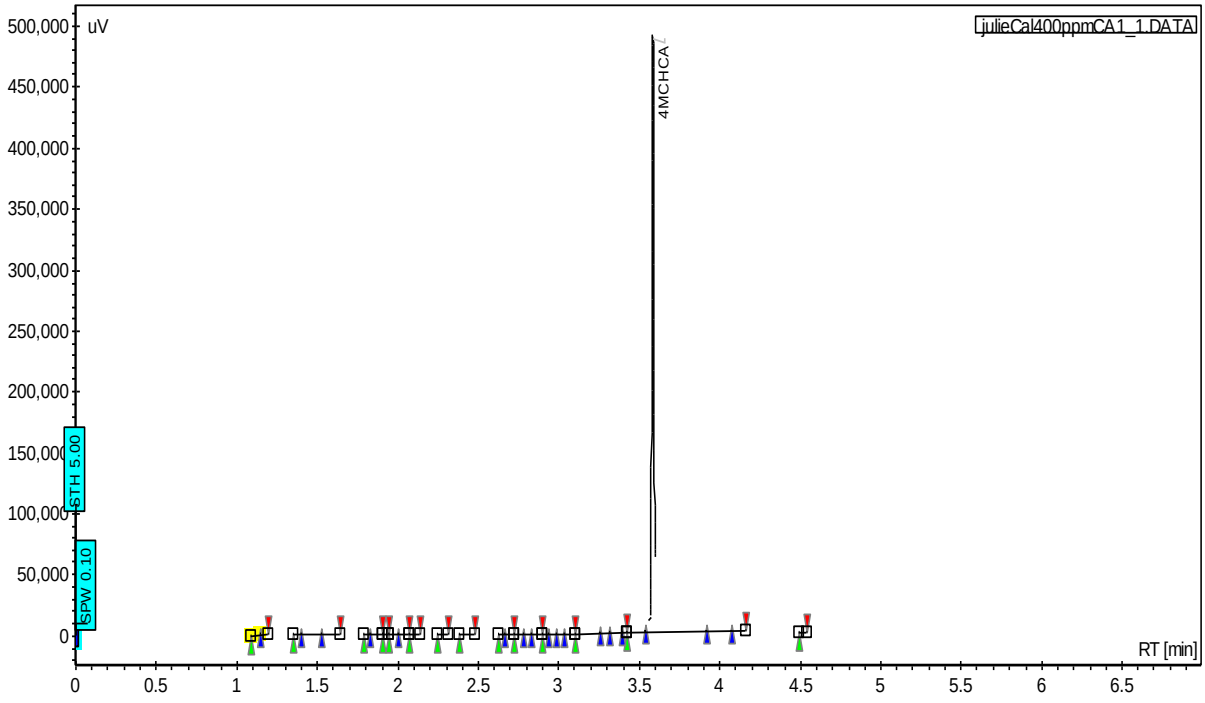

Figure B.2. The representative GC/FID chromatogram of trans-4MCHCA investigated.

APPENDIX C

Sample Calculations

The performance of continuous stirred tank reactor (CSTR) and biofilm were evaluated based on dilution rate, residence time of the reactor, trans-4MCHCA loading rate, trans-4MCHCA removal rate, and trans-4MCHCA removal percentage. The dilution rate is calculated by flow rate over the volume of the reactor. For example, at flow rate of 0.49 mL h^{-1}, with working volume of 200 mL, CSTR had dilution rate of 0.0024 h^{-1}.

$$D = F / V \quad \text{(C.1)}$$

Hydraulic residence time (HRT) of CSTR is the inverse of dilution rate, so CSTR at dilution rate of 0.0024 h^{-1} had hydraulic residence time of 416.7 h.

$$HRT = 1 / D \quad \text{(C.2)}$$

Loading rate is calculated by multiplying the initial feed concentration of substrate with dilution rate.

$$\text{Loading rate} = (S_i * D) \quad \text{(C.3)}$$

Removal rate of the substrate is calculated by multiplying the difference between initial feed concentration of substrate and residual substrate concentration with dilution rate.

$$\text{Removal rate} = (S_i - S_r) * D \quad \text{(C.4)}$$

Finally, removal percentage of substrate is calculated by dividing the difference between initial concentration of feed substrate and residual substrate concentration with initial substrate feed concentration, and then multiplying by 100%.

$$\text{Removal Percentage} = ((S_i - S_r) / S_i) * 100\% \quad \text{(C.5)}$$

APPENDIX D

Negative Control Experiment Data

Figure D.1 and Figure D.2 show the data from duplicate control experiments of both 250 and 500 mg L^{-1} of trans-4MCHCA with their corresponding nitrate of 620 and 1,240 mg L^{-1}, respectively to demonstrate that abiotic degradation did not occur.

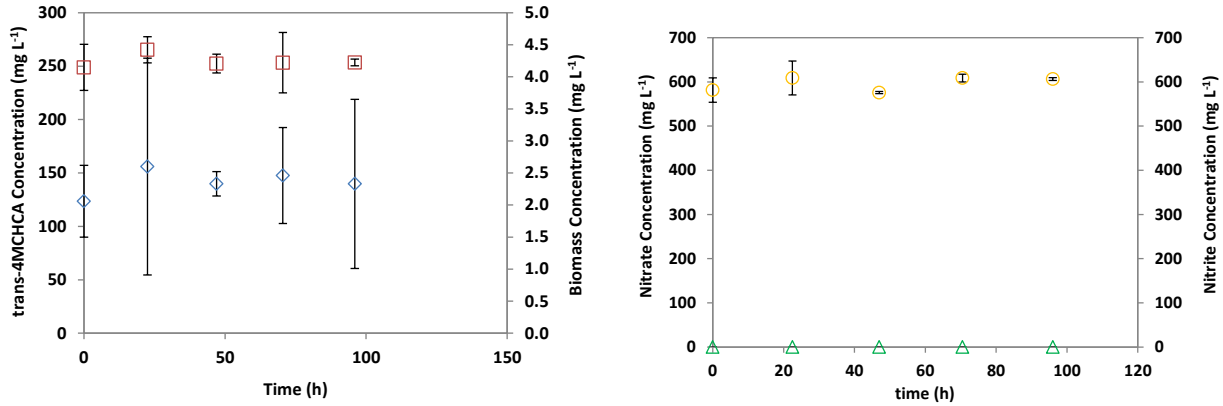

Figure D.1. Negative control of duplicate experiments with 250 mg L^{-1} of trans-4MCHCA and 620 mg L^{-1} of nitrate in the absence of inoculum.

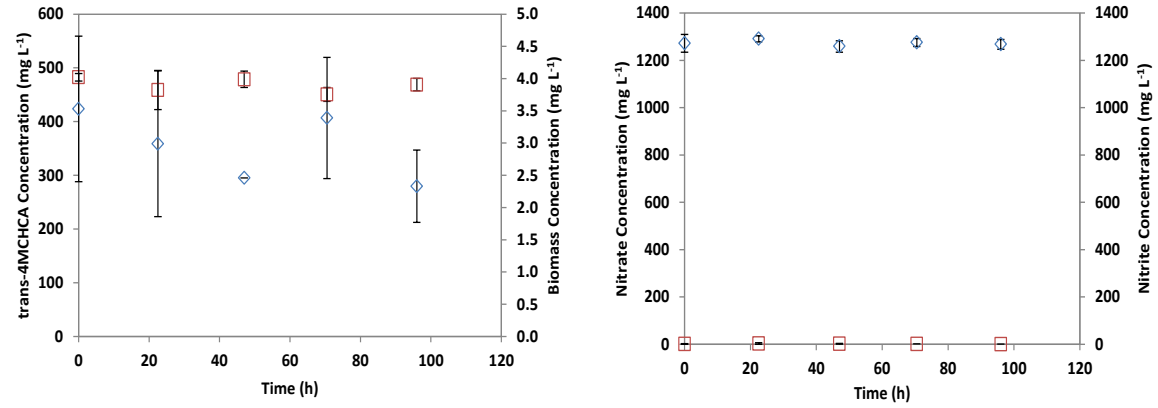

Figure D.2. Negative control experiment with 500 mg L^{-1} of trans-4MCHCA and 1,240 mg L^{-1} of nitrate in the absence of inoculum.

The negative control of duplicate experiments with 250 mg L^{-1} of trans-4MCHCA and 10% inoculum, with the absence of nitrate as the electron acceptor, show that biodegradation of trans-4MCHCA was coupled with denitrification process (Figure D.3).

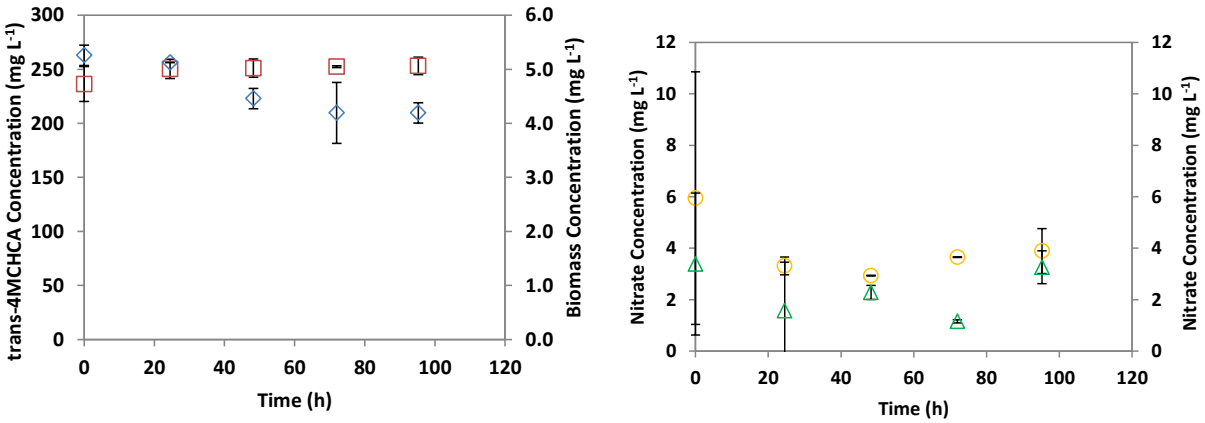

Figure D.3. Duplicate negative control experiments with 250 mg L^{-1} trans-4MCHCA, 10% (v/v) inoculum, and no nitrate.